Development of a two phase enzyme reactor for conversion of hydrophobic substrates

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Award date:
1992

Awarding institution:
University of Bath

DEVELOPMENT OF A TWO PHASE ENZYME REACTOR FOR CONVERSION OF HYDROPHOBIC SUBSTRATES.

Submitted by Andrew Robert Booth

for the degree of PhD

of the University of Bath

1992

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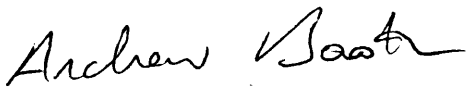


Andrew Booth

ACKNOWLEDGEMENTS.

I would like to extend my special thanks to both of my supervisors, Dr. John Hubble and Dr. Colin Soper, for their professional advice and support. I would also like to extend my gratitude to all the technical staff of the School of Chemical Engineering for their technical advice.

ABSTRACT

A model enzyme system was used to investigate the potential of a novel, two-phase, membrane bioreactor for the conversion of hydrophobic substrates where the biocatalyst also has a requirement for a hydrophilic coenzyme. The model system chosen was the conversion of octan-1-ol to octyl aldehyde by horse liver alcohol dehydrogenase (HLADH, E.C.1.1.1.1.).

The enzyme was immobilised, by adsorption, to Millipore Minitan® microfiltration membranes. The function of the membrane was to act as the enzyme support and to separate an organic phase consisting of octan-1-ol dissolved in 2,2,4 trimethyl pentane from an aqueous phase consisting of the coenzyme (NAD^+) dissolved in buffer.

A small glass, two-phase reactor was developed to examine the kinetic behaviour of enzyme immobilised to 12 cm^2 membrane circles with a nominal pore size of 0.22 μm. Results of initial reaction rate determinations were compared to those obtained using soluble enzyme in a single-phase, aqueous system. Reaction rates with the immobilised enzyme were approximately 100-fold lower than those for soluble enzyme, this was attributed to denaturation of the enzyme upon immobilisation. Results showed deviation of the immobilised enzyme's kinetics from Michaelis-Menten behaviour and differences from soluble enzyme kinetics with respect to variation of pH and ionic strength. These effects were attributed to a

combination of internal mass transfer limitations and partitioning of charged substrate (NAD^+) and product (H^+) in the immobilised enzyme system.

A second, larger-scale (60-300 cm^2 membrane area), batch reactor was developed to investigate coenzyme regeneration strategies to allow the use of less than stoichiometric amounts of coenzyme. Two coenzyme regeneration systems were investigated which used enzymatic reduction of acetaldehyde to ethanol to oxidise NADH to NAD^+. The first system used the immobilised HLADH to catalyse the regeneration reaction as well as the conversion of octan-1-ol. The second system used yeast alcohol dehydrogenase (YADH) dissolved in the aqueous phase to support coenzyme regeneration. Comparative batch reactions produced high fractional yields (approximately 90% conversion) of octyl aldehyde in both cases. However, no increase in reaction rate was observed due to incorporation of YADH into the system.

The YADH system was used to regenerate coenzyme over, 5, 24 hour batch reactions. In this system, stability of the immobilised HLADH was found to be enhanced. This effect was attributed to a combination of operational stability and stabilisation due to continuously catalytic state of enzyme. The total turnover number for the coenzyme in this system was 11.72 (the maximum values possible under the reaction condition was 20).

The reactor was demonstrated to be technically feasible in principle and low enzyme activties and mass transfer limitation of reaction were identified as important problems to be overcome for the reactor design to be of commercial interest.

CONTENTS

CHAPTER 1

BIOTRANSFORMATIONS IN AQUEOUS, ORGANIC SOLVENT, TWO-PHASE SYSTEMS

1.1 INTRODUCTION

Conventionally biocatalytic reactions have been carried out in wholly aqueous environments or less commonly in systems containing a proportion of water-soluble organic solvent (Butler 1979). This restriction has limited the applicability of enzymes to commercial processes such that only about 30 of the 2000 or more known enzymes are used in multi-tonne quantities. Of these more than 80% are simple hydrolases with no cofactor requirement (Godfrey and Reichelt 1983).

Other classes of enzymes of potential interest eg. oxidoreductases have not been significantly exploited on an industrial scale. These enzymes have been shown to have a tremendous potential for catalysis of stereo-specific and regio-specific reactions of commercial importance (Carrea 1984). However, as many of the substrates or products of interest have very low solubilities in aqueous media enzymes are still only rarely used in organic chemical syntheses.

Conversely, a number of whole cell preparations are used commercially for

the transformation of poorly aqueous soluble substrates; particularly for the production of steroid derivatives. Even so, the elaborate batch processing techniques that are often required can exclude the use of a biocatalyst in favour of a chemical route (Sedlaczek 1988).

However, within the last fifteen years a wide range of biocatalytic reactions have been shown to be effective in biphasic, aqueous-organic solvent systems and this has lead to a great deal of research interest in this area.

1.1.1 Definition of Reaction Type and Reaction Medium Characteristics

Two-phase reactions usually involve an aqueous phase containing biocatalyst and sometimes substrate(s), this can also include a coenzyme regeneration system for isolated enzymes. The aqueous phase is contacted with an immiscible organic solvent phase which contains, or is, the substrate. Reaction occurs at the phase interface or by diffusion of the organically soluble substrate into the biocatalyst containing aqueous phase. Upon reaction products are liberated and partition between the phases depending upon their relative solubility preferences.

As an example, the first report of such a reaction was given by Cremonesi et.al. (1973).

testosterone + NAD^+ ⇌ androst-4-ene-3,17-dione + NADH + H^+

The reaction was carried out in a two-phase emulsion with the enzyme (3,17-β-hydroxysteroid dehydrogenase) and coenzyme located in the aqueous phase. The steroid substrate was contained in the organic phase. Kinetic constants of the enzyme were found to be similar to those of the equivalent reaction in an aqueous system.

Often the biocatalyst is immobilized to a support eg. Sepharose (Cambou and Klibanov 1984). The aqueous phase content may be as low as 0.02% ie. a 99.98% organic solvent environment! Evidently such systems can bring very high concentrations of poorly aqueous soluble substrates into close proximity with the biocatalyst. Accordingly, reaction rate increases over wholly aqueous systems have been observed (eg. Buckland, Dunhill and Lilly 1975). In addition such systems can afford a number of other benefits which will be discussed in detail later.

Classification of aqueous, organic solvent biphasic systems has been carried out by Lilly (1982,1983) and Halling (1987). However, for the purposes of this discussion the systems will be defined by the following criteria:

1) whether there is a surfactant used at the phase interface;

2) whether cells/organelles or enzymes are used.

The use of enzymes dispersed in organic solvents has been discussed by Inada et.al.(1986) and will not be dealt with here. Within the criteria defined above

selective topics will be discussed on the basis of particular relevance to this thesis and discovery merit within the general field.

1.2 SYSTEMS WITH SURFACTANT AT THE PHASE INTERFACE

In normal micelles microdroplets of immiscible organic solvent are surrounded by surfactant in a continuous aqueous phase. In the complimentary situation we have reverse micelles. Reverse micelle systems have received much attention in the literature. Essentially in these systems minute aqueous pools of 2-20 nm diameter are surrounded by an immiscible continuous phase, eg. isooctane. At the interface is deposited an amphiphilic surfactant layer, eg. di(2-ethyl-hexyl) sodium sulphosuccinate (more commonly termed AOT). The surfactant stabilizes the microemulsion. The hydrophilic "heads" of the surfactant lie in contact with the aqueous phase and the hydrophobic "tails" point out into the organic phase. (see Figure 1.1). Due to their small size the interfacial areas obtained in such systems are of the order of $100m^2/ml$ (Luisi and Wolf 1982).

Reverse micelles can be simply and quickly formed by several methods. Once formed a microemulsion is homogeneous, transparent and stable. Hence many of the characteristics of these systems can be elucidated by conventional spectroscopic means. The most important criterion in the characterisation of these systems is the molar ratio of aqueous phase to surfactant, ie. $W_0=[H_2O]/[surfactant]$. This parameter controls the diameter and so the volume of the micelles. Evidence gained from

sedimentation experiments using ultracentrifugation suggests the distribution of micellar diameters for any given value of W_0 is close to uniformity (Luisi and Wolf 1982). Further, micelles either contain a single enzyme molecule or none.

As the value of W_0 is increased from 0 to approximately 5 (values vary with chemical composition) the added aqueous phase becomes associated with the polar "heads" of the surfactant molecules. Biocatalysts added to such systems typically demonstrate low activity because too little aqueous phase is present to fully hydrate the biocatalyst and form a continuously spherical micelle. Above W_0 values of 6-8 micelles form which encapsulate the biopolymer within a "water-pool" .

The proposed structure across a section through the radius of a reverse micelle is: organic phase; surfactant; peripheral aqueous layer associated with surfactant; free "water-pool". With further increases in W_0 the peripheral layer thickness remains unchanged and the "water-pool" diameter expands (Luisi 1985).

1.2.1 Enzyme Activities in Reversed Micelles

Enzymes reportedly active in reverse micelles include: proteases; other hydrolases eg. lysozyme, lipase; oxidoreductases; and oxygenases. Optimal activities are typically found at W_0=10-15, see Figure 1.2 for example. This may represent a condition whereby the enzyme is wholly enveloped by the "water-pool" but, distances

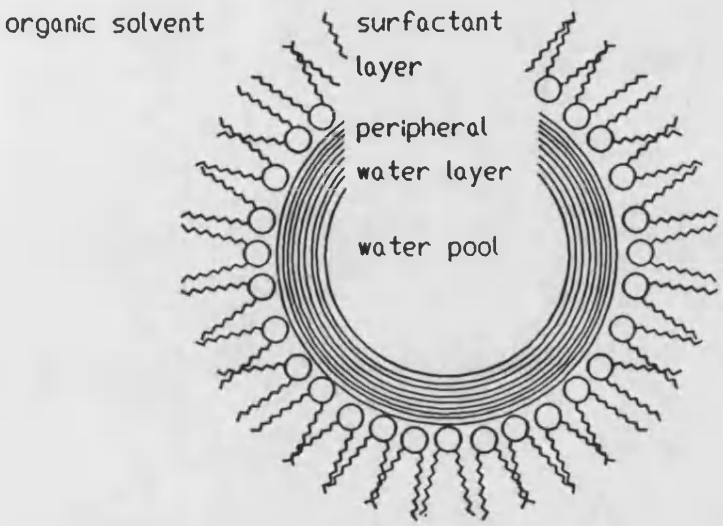


Fig 1.1 Diagram of a Reverse Micelle

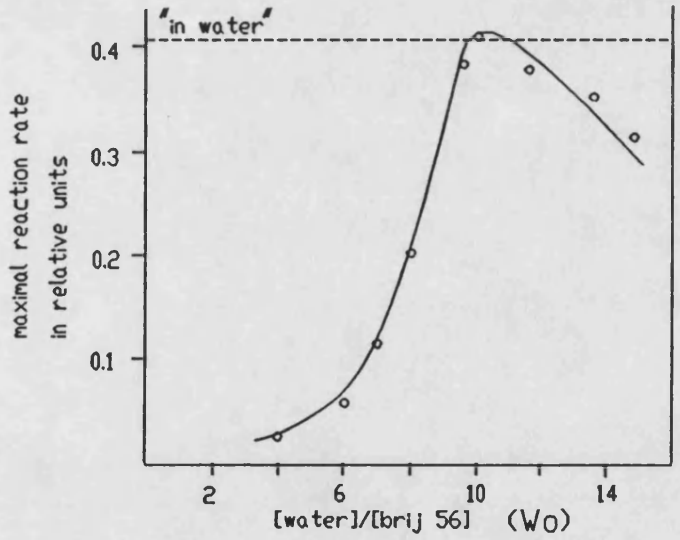


Fig 1.2 Change in Maximal Velocity of Pyrophosphate Hydrolysis with Wo.
0.2 M brij 56, 0.05M Tris-HCl pH 7.0, cyclohexane. At 26 C. Overall [pyrophosphatase]= 0.003 uM, [pyrophophate]= 0.0125mM. From Martenik et. al. 1981.

from the active sites to the bulk organic phase are minimal (Luisi and Steinman-Hofmann 1987).

The magnitude of the activities reported varies from values greater than those reported for native enzymes to completely inactive. A majority of enzymes are reported with activities lower than those for the native enzyme in aqueous systems. In many cases this is probably due to the use of sub-optimal conditions. For example, Martinek et.al. (1982) examined the activity of horse liver alcohol dehydrogenase on aliphatic, primary alcohols of varying chain lengths in an AOT/isooctane system. The substrate specificity changed from octan-1-ol, in a wholly aqueous system, to butan-1-ol in the reverse micelle system. The effect was independent of variations in W_0 and was attributable to the greater compatibility of butan-1-ol with the surfactant. Other workers have supported this view (Hilhorst et.al. 1984).

A 20-fold increase in activity over a wholly aqueous system was reported for the horse radish peroxidase catalysed oxidation of pyragallol in an AOT/isooctane system with $W_0=10$ (see Figure 1.3). This was due to the removal of substrate inhibition present when the reaction was attempted in wholly aqueous media (Martinek et.al., 1981).

Menger and Yamada (1979) demonstrated the superactivity of α-chymotrypsin in a heptane/AOT system. At optimal pH and $W_0=25$, Kcat (moles of product/unit time / mole enzyme) was found to be double that for a comparable aqueous system. The substrate was N-acetyl-L-tryptophan. Barbaric and Luisi (1981) also reported the

enhanced activity of this enzyme on N-glutaryl-L-phenylalanine p-nitroanilide in an AOT/isooctane system but, at low W_0 values (eg. 9). Values of Kcat were upto 6-fold higher than in a compared aqueous system. These workers also demonstrated the enhanced stability of the enzyme in reversed micelles. The increased stability and activity were explained by a local lowering of the dielectric constant with respect to the micellar "water" leading to an increase in enzyme tertiary structure folding. Hence, a more rigid conformation was favoured which coincidentally was the more active form. In addition, as only one molecule of enzyme occupied each micelle autolytic effects were minimised.

Hillhorst, Veegar and Laane (1983) have reported the site specific oxidation of a keto-steroid in reverse micelles. Molecular hydrogen was transferred by a hydrogenase to reduce methyl viologen (MV). The reduced MV is then used to reduce NAD^+ to NADH using lipoamide dehydrogenase. This provides 20 β-hydroxysteroid dehydrogenase with reduced cofactor for reaction with the keto-steriod progesterone (see Figure 1.4). The hydrogenase was reported as more stable in the reverse micelle than in aqueous solution.

The increased stability, over wholly aqueous systems, of enzymes in reverse micelles has been reported by other workers (eg. Erjomin and Metelitz 1983) though this is not a universal phenomenon (Luisi and Steinmann-Hofmann 1987). Martinek et.al. (1981) stated that an AOT/isooctane solution (W_0 approx. 5.5) of chymotrypsin remained active for over two years. Neither the degree of activity remaining nor the full storage conditions were reported. No reason was put forward for this effect.

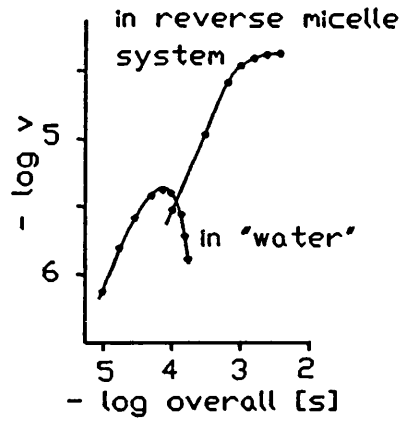


Fig 1.3 Initial Rate of Peroxidase Oxidation of Pyrogallol. At 26 C , 0.1M AOT, 2%(v/v) 0.02M phosphate/borate/acetate buffer, pH 7.0, octane overall [enzyme]= 0.003uM, [hydrogen peroxide]= 2.5mM
From Martenik et. al. 1981

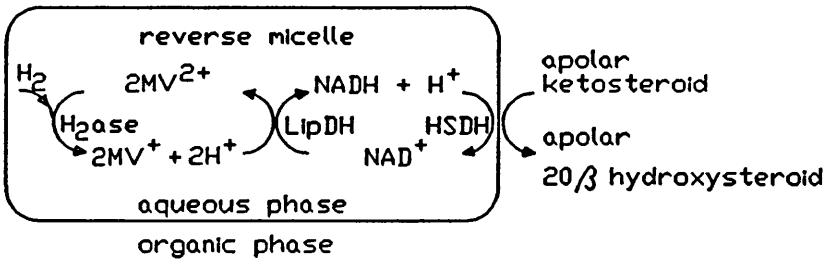


Fig 1.4 Apolar Steroid Reduction Coupled to NADH Regeneration in a Reverse Micellar Medium. LipDH= dihydrolipoamide, HSDH= 20 β -hydroxsteroid dehydrogenase
H ase= hydrogenase, MV= methyl viologen
From Hilhorst et. al. 1983

As can be seen from the above examples the use of biocatalysts in reverse micelles can produce a range of novel properties many of which are system specific.

1.2.2 Cells/Organelles Within Reverse Micelles

Recently bacteria, plasmid DNA and mitochondria have been solubilised in reverse micelle systems (Hochkoeppler and Luisi 1989). These studies have shown the retention of significant biological activity in all cases. No specific uses have been forwarded for these systems at this time.

1.3 SYSTEMS WITH NO SURFACTANT AT THE PHASE INTERPHASE

Within this group of systems there is a good deal of diversity both in system design and in the reactions undertaken. In terms of system design the earliest types were emulsion systems such as the example of Cremonesi et.al. (1973) discussed earlier. Since then systems have diversified with tendencies to immobilise the biocatalyst and to reduce the aqueous phase volume being prominent trends.

Immobilisation of the biocatalyst in two-phase systems affords many benefits similar to those gained upon biocatalyst immobilisation in aqueous systems, for example; easy reuse of the biocatalyst, minimal leakage of the biocatalyst from the system and the potential for continuous operation. However, other advantages may

become apparent when biocatalysts are immobilised in aqueous-organic two-phase systems. For example, Bar et.al. (1987) investigated the use of organic solvents as extractive agents to remove a toxic product (lactic acid) from fermentations of L. delbrueckii. When n-dodecanol was used as the solvent in the extractive fermentation cell growth was completely inhibited. Subsequently they demonstrated that the low concentration of n-dodecanol partitioning into the fermentation broth was not responsible for the inhibition of growth and hence, the presence of the bulk organic phase was surmised to be responsible. This was demonstrated by the fermentation running to completion when the cells were immobilised in a carrageenin gel and were not accessible by the bulk organic phase.

Designing two-phase systems with low water contents evidently allows for a reduction in reactor volumes. However it also affords other more interesting benefits. When the reaction:

$$A + B \rightharpoonup C + H_2O$$

is considered then it is apparent that in an aqueous system where water is present at 55.5 M (i.e. a water activity of 1) the forward reaction is thermodynamically unfavourable. In aqueous-organic systems with water activities below 1 an equilibrium shift in favour of products occurs. This technique has been used to boost the yield of products for many reactions.(eg. Cambou and Klibanov 1984, Knox and Cliffe 1984, Yamane, Hoq and Shimizu 1985). This effect has been modelled by Martinek et.al. (1981b) and re-examined by Halling (1984).

Klibanov (1989) and Zaks and Klibanov (1984, 1985) were instrumental in taking systems with reduced volumes of aqueous phase to their extreme. The group demonstrated the transesterification activity of powdered porcine pancreatic lipase in organic solvents. The overall water content of the system was below 0.02%. It was considered that the enzyme had an "essential water layer" bound to its surface and as the solvent was immiscible and of low polarity it was unable to "strip-off" the water layer.

In such a system the lipase was shown to have a half life of twelve hours at 100°C (1984). This is initially a surprising result but it can be rationalised easily as the few water molecules present are considered to be rigidly bound to the enzyme molecule and stabilize it at elevated temperatures. The situation is analogous to that found on examination of the rationale for the thermostability of some bacterial spores. Here, again, the tiny amount of water present is rigidly bound.

Another phenomenon observed was the ability of the enzyme particles to "remember" the pH of the solution of buffer they were prepared from i.e. the activity of enzyme particles in the organic solvent system was found to be dependant on the pH of the solution from which they were precipitated and dried. Hence, pH can easily be optimised in these systems.

1.3.1 Enzyme Activities in Aqueous-Organic, Two-Phase Systems Without a Surfactant Interface

Amongst the syntheses carried out, steroid biotransformations, peptide syntheses and esterifications have been favoured. Carrea, Cremonesi and co-workers have carried out an extensive series of steroid biotransformations in biphasic systems which mainly comprise of regio-specific reductions and oxidations (e.g. Carrea 1984, Carrea 1986, Carrea et.al. 1984, Carrea 1979, Carrea and Cremonesi 1987). The oxido-reduction reactions used were often coupled to a coenzyme regeneration system. Both NADH and NADPH coenzymes were employed with a range of regeneration systems.

The use of $NAD^+(P)/NAD(P)H$ requiring enzymes can be particularly advantageous in an aqueous-organic, two-phase system as nicotinamide coenzymes are practically insoluble in non-polar organic solvents (Martinek et.al. 1981b). So, where coenzyme regeneration is used in a system where the target product partitions selectively into the organic-phase, then an equilibrium shift in favour of products will occur (Okahata and Ijiro 1988). Favourable equilibrium shifts can be enhanced by removal of one of the end products of coenzyme regeneration. In a single-phase, aqueous system Wandrey et.al. (1986) have used formate dehydrogenase (FDH) to regenerate NADH via the oxidation of formate to carbon dioxide and water. Removal of carbon dioxide was simply accomplished by degassing.

A full range of techniques have been used to investigate peptide synthesis in

aqueous-organic, two-phase systems. These include: emulsions (Semenov, Berezin and Martinek 1981); the use of solid enzymes in anhydrous solvents (Ooshima, Mori and Harano 1985); immobilisation of the enzyme to aqueous phase containing particles (Oyama et.al. 1981, Riva et.al. 1988); and the use of enzymes immobilised to particles with an aqueous phase water activity defined as below unity (Cassels and Halling 1989). Often the peptide products solvate preferentially in the organic phase relative to the starting amino acids. So, an equilibrium shift in favour of synthetic products occurs. Khmel'nitskii et.al. (1984) found that upon optimisation their synthesis of N-actyl-L-tryptophanyl-L-leucine amide from N-acetyl-L-tryptophane and L-leucine amide in an aqueous-ethyl acetate emulsion could yield 100% product. The protease used was α-chymotrypsin.

An interesting system is that of Okahata and Ijiro (1988) who immobilised thermolysin on to the outside of nylon capsule membranes of 2mm diameter containing buffer. These were dispersed in a chloroform continuous phase to catalyse the coupling of an L-aspartic acid derivative and L-phenyl alanine methyl ester to yield the corresponding dipeptide derivative. The system showed decreased thermolysin activity but greatly increased operational stability over controls. Only 20% activity was lost after nine, 24 hour syntheses.

Cassels and Halling (1989) have attempted peptide synthesis in a system where the aqueous phase water activity was below unity. As peptide formation liberates water by elimination, theoretically, a low water environment should shift the reaction equilibrium in favour of synthetic products. However, so far these workers have met

with little success.

Schutt et.al. (1985) have used substilisin immobilised onto a resin to prepare D-arylglycines in an aqueous-organic, two-phase system. The D-arylglycines are valuable as side chains for semisynthetic antibiotics. In this system the organic phase acted as a reservoir for D,L-amino acid methyl esters. The resin bound substilsin was mixed into an emulsion of the organic phase (containing the reactants) and an aqueous phase. The substilisin hydrolysed the L-amino acid methyl ester to the corresponding amino acid which accumulated in the aqueous phase. The pure L-amino acid could then be recovered from the aqueous phase.

Klibanov's group have used proteases to perform transesterifications in organic solvent (Riva et.al. 1988, Zaks and Klibanov 1986). In one group of experiments solid substilisin was used to regioselectively acylate sugar moieties in dimethylformamide. The general reaction was:

$$\text{R COOR'} + \text{Sugar} \rightleftharpoons \text{Sugar monoester} + \text{R'OH}$$

Regioselectivity of the reaction was often greater than 95%. Futhermore this reaction was used to introduce amino acids into sugars and the butyryl moiety into compounds with a non-glycon portion, for example; adenosine, riboflavin and uridine. Earlier work by Therisod and Klibanov (1986) had used a lipase to form acylated sugars in pyridine from esters and sugars via transesterification.

Zaks and Klibanov (1985) have carried out a range of other transesterifications in organic solvents catalysed by lipase enzymes. For example, reaction between tributyrin and heptanol was carried out in 99.8% 2-pentanone. Under these conditions several lipases demonstrated activities not observed in aqueous media including some esterifications.

Esterifications readily occur in these systems as the water content is low. From the general reaction:

$$RCH_3\text{-}OH + HOOC\text{-}R' \rightleftharpoons RCH_3\ COOCR' + H_2O$$

it can be seen that by controlling the water content the reaction equilibrium can be shifted to favour synthetic or hydrolytic products. A series of articles have discussed the use of lipase enzymes immobilised to microporous membrane supports (Hoq et.al. 1985a, 1985b, 1986, Koizumi et.al. 1987, Yamane et.al. 1985, 1986a, 1986b, 1987). In many cases use of the system for the hydrolysis or synthesis of fats was directed by controlling the level of hydration of the system.

Cambou and Klibanov (1984) and Langrand et.al. (1985) have both discussed the use of lipase catalysed esterifications as a means to resolve optical enantiomers. As an example, Langrand's group reacted a lipase with racemic α- substituted cyclohexanols (eg. menthol) and a fatty acid in an organic solvent (hexane or heptane). The synthesis yielded the (+) ester and the unreacted (-) cyclohexanol derivative. These compounds were then separated by distillation. Reduction of the

(+) ester was then possible to give overall pure (+) and (-) forms of the cyclohexanol derivative.

1.3.2 Catalysis by Cells/Organelles in Aqueous-Organic, Two-Phase Systems Using no Surfactant at the Interface

Cell based systems have not received as much attention in the literature as isolated enzyme systems. Use of a cell based reaction may be advantageous if the organism can carry out a complex serial reaction or if the isolated enzyme proves unstable. A further advantage is that cells are much simpler, quicker and hence cheaper to obtain than enzymes. A potential problem of cell based reactions is the accumulation of side products.

Chiefly cell based aqueous-organic, two-phase reactions have been of two types: i) extractive fermentations of growing cultures to boost yields of primary metabolites; and ii) systems utilising non-viable or stationary phase cultures.

Bell et.al. (1978) have used freeze-dried Rhodococcus arrhizus mycelia to carry out esterifications and glyceride synthesis in organic solvents. Glyceride synthesis from glycerol and oleic acid was carried out in acetone. The main products were mono- and diglycerides. By adding a molecular sieve to absorb water produced by the reaction, yields were significantly improved. However, excessive dehydration did reduce product yields. Later Knox and Cliffe (1984) used a freeze-dried R.

arrhizus mycelium in a packed bed reactor. Heptane containing oleic acid and cetyl alcohol was circulated through the bed to produce cetyl oleate. A molecular sieve was inserted downstream of the reactor to remove the water produced before recycling through the reactor. In this manner the reaction rate was maintained at a constant level for 16, 24 hour runs. It was also found that the reaction rate could be increased by raising the ratio of fatty acid to alcohol. Because of this discovery it was reasoned that the alcohol competitively inhibited fatty acid access to the catalytic site.

Ueda et.al. (1986) used R. equi to investigate the oxidation of higher alcohols in octane. Even-numbered, C4-C14, primary alcohols were initially oxidised to the corresponding carboxylic acids and on further reaction the corresponding esters were formed. Direct esterification of tetradecanol and tetradecanoic acid was also shown to occur using acetone dried cells in octane. The system was "practically free from water" and similarly to Zaks and Klibanov (1984) enhanced thermal stability was demonstrated by reaction rate increases upto 70°C. "Wet cells" showed no esterification activity at this temperature.

Buckland et.al. (1975) demonstrated the conversion of cholesterol to cholest-4-ene-3-one by a Nocardia sp. in a range of solvents, principally carbon tetrachloride. Specific yields were 6.25 g/l/hour in the two-phase system and 0.04 g/l/hour in the wholly aqueous system. In batch experiments seven runs were carried out in 69 hours after which 52% of the original activity remained.

Epoxidation by Pseudomonas oleovorans has also been investigated. Schwartz

et.al. (1972) studied the conversion of 1,7 octadiene to 7,8 epoxy-1-octene and 1,2-7,8 diepoxyoctane in the presence and absence of 20% (v/v) cyclohexane. In the presence of cyclohexane the epoxy product partitioned selectively into the solvent phase keeping aqueous phase concentrations low at approximately 0.4 g/l of 7,8 epoxy-1-octene. In the absence of cyclohexane accumulation of product inhibited further reaction above 0.8 g/l . Correspondingly, conversions after 100 hours were approximately 90 and 15 mol percent in the presence and absence of cyclohexane, respectively.

Extractive fermentations have been used to boost yields of ethanol from Saccharomyces cerevisae fermentations. Minier and Goma (1982) used a continuous column reactor with yeast immobilised to a porous support. Dodecanol was used as the extractant, lower primary alcohols were found to be inhibitory to cells. The two-phase system showed total utilisation of 409 g/l glucose and produced 5.7 times more ethanol than the control fermentation which utilised 200 g/l glucose. The reactor was operated for 1.5 years and was considered as a generic design.

Cho and Shuler (1986) and Efthymiou and Shuler (1987) demonstrated an extractive ethanol fermentation where cells and medium were separated from the extractant solvent, tributyl phosphate, by a microporous membrane. In this manner the toxic effects of the solvent were overcome. Tributyl phosphate was described as an ideal solvent for ethanol extraction.

Finally, Bang et.al. (1983) have used an organic solvent as a substrate

reservoir in the production of L-tryptophan from indole and D,L-serine by an Escherichia coli strain. High concentrations of indole inhibited tryptophan synthetase activity. Hence, reaction in a system containing 20% (v/v) toluene enhanced productivity 4 times relative to a wholly aqueous control. The indole was preferentially solubilised by toluene keeping the aqueous phase concentration substantially reduced.

1.4 MEDIUM ENGINEERING FOR AQUEOUS-ORGANIC, TWO-PHASE, BIOCATALYSIS

Laane et.al. (1985, 1987a, 1987b) have attempted to understand the factors of key importance to enzyme activity in organic solvent systems. Reverse micelle systems were used as a model. Initially, a cosurfactant, hexanol was used in addition to CTAB (cetyl trimethyl ammonium bromide) and isooctane to provide a medium with a variable surfactant polarity in which the 20 β-hydroxylation of progesterone and prednisone was carried out. In the treatment of results the group used log P (partition coefficient) values as a measure of each components polarity. They stated that: solvents with log P < 2 were unsuitable for use with biocatalysts; solvents with a log P value between 2 and 4 could be either suitable or unsuitable for use with a biocatalyst and; solvents with a log P value of 4 or more were the solvents of choice for use with a biocatalyst. Essentially, the lower the log P value the more solvent will be dissolved in the aqueous phase and the greater the denaturation effect of the solvent on the biocatalyst. Further, by ensuring the difference between log

P(interphase) and log P(substrate) was as small as possible substrate compatibility with the surfactant layer was maximised and substrate was considered to be concentrated in the surfactant layer. Enzyme activity was shown to increase as this parameter was varied towards 0. Similarly, by ensuring the difference between log P(continuous) phase and log P(substrate) was maximal substrate concentration in the organic phase was kept low and partition of the substrate into the surfactant layer was favoured. Enzyme activity was shown to increase as this parameter was increased. Some of their results are reproduced as Figure 1.5. As can be seen the effect of varying these two parameters to their optimal values was additive. A simplified set of rules was applied to aqueous, organic solvent systems using no surfactant.

Recently Osborne et.al. (1990) showed the cell membrane associated, 11 α-hydroxylase activity of whole, viable cells of Rhizopus nigricans in both aqueous-organic, two-phase systems and in aqueous systems containing a proportion of organic solvent. It was demonstrated that loss of activity depended upon the establishment of a concentration of organic solvent above the "critical membrane concentration" (CMC) in the cell membrane. The (CMC) was shown to be independent of the solvent type. Activity was also lost due to "gross phase effects" (for an example see the work of Bar et.al. (1987) outlined in Section 1.3). By deriving an expression giving the log P value of the solvent in the membrane, solvents were categorised into two groups: a) solvents producing loss of activity due to the solvent being able to attain cell membrane concentrations above the CMC and; b) solvents unable to attain a cell membrane concentration above the CMC with which

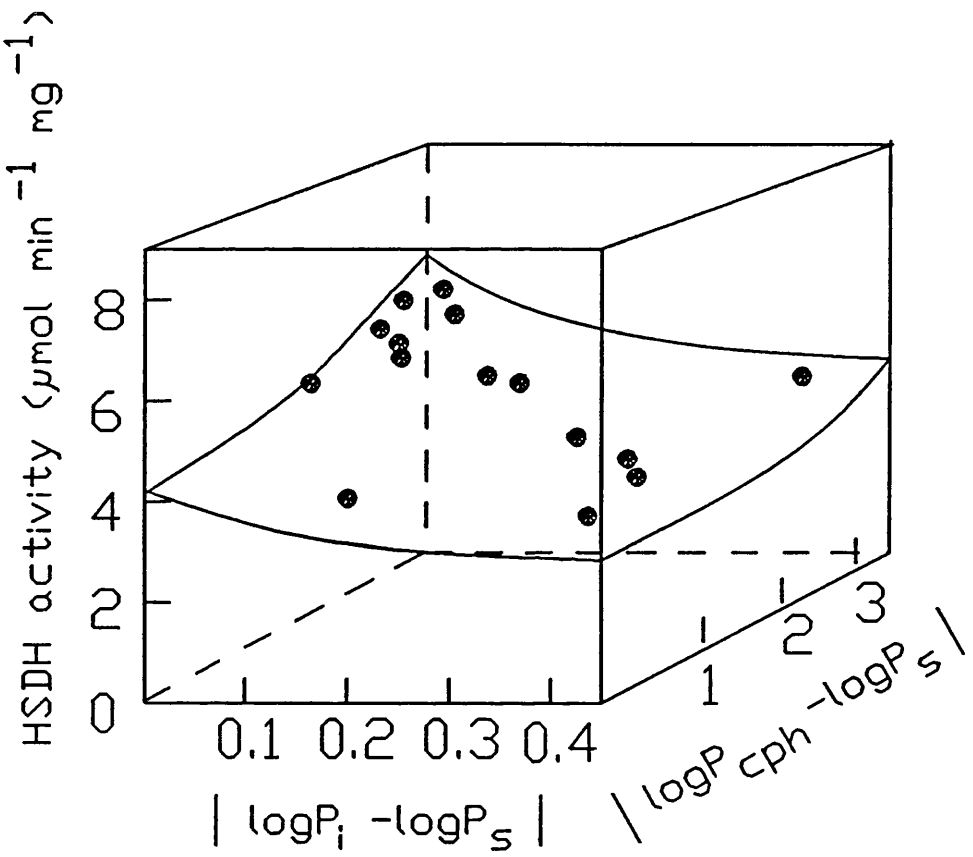


Fig 1.5 Optimisation of 20β-Hydroxysteroid Dehydrogenase Activity in a Reverse Micellar Medium. S= substrate (prednisone), i= interphase (hexanol/CTAB), cph= continuous phase (varied). From Laane et. al. 1987

activity was only lost due to "gross phase effects". Obviously, group b were the solvents of choice.

1.5 REACTORS FOR AQUEOUS-ORGANIC, TWO-PHASE BIOCATALYSIS

Few reactor designs have been forwarded in the literature. As the field has only been in existence for around fifteen years most studies have been fundamental. However, of the designs that have appeared some are at an advanced stage of development. Also, there is a mixture of traditional and novel approaches. Typical of the former group are stirred tank reactors (Van der Meer et.al. 1986) and packed bed reactors (Knox and Cliffe 1984). Of the latter group designs are mainly membrane based reactors (Hoq et.al. 1985a, 1985b, 1986, Katsunori et.al. 1986, Koizumi 1987, Sakata et.al. 1986, Taylor et.al. 1986, Yamane et.al. 1985, 1986a, 1986b). Most of the membrane reactors cited utilise the membrane to keep the two phases separate, thus eliminating the need to separate the phases downstream, and to immobilise the biocatalyst.

Luithi (1984) has suggested a membrane reactor for reverse micelles. Reverse micelles containing α-chymotrypsin and an amino acid were introduced into the lumen of a hollow fibre reactor. Organic solvent containing a dipeptide methyl ester was circulated within the fibre lumen and throughout the external fibre spaces. The reactor produced a low yield of the tripeptide (10%). This was attributed to the unfavourable

partitioning of reagents into the relatively large organic phase volume. The proposed advantage of the design was the ability to separate product from the enzyme by removing product containing solvent from the external fibre spaces. The reverse micelles were effectively entrapped within the fibres.

A number of reports have dealt with the use of lipase enzymes immobilised within the pores of membranes. Typically, the membrane also separates the phases. The reactor configuration is shown schematically as Figure 1.6. Interestingly, the lipase is immobilised by adsorption to the phase interface within the membrane pores. So far, only enzymes which act at phase interfaces have been used in this manner. Usually 0.2 micron cut-off, hydrophobic membranes of high porosity have been used, hence, the organic phase has a natural tendency to permeate through the membrane and form emulsion droplets in the aqueous phase. This effect has been counteracted by applying a slight positive pressure to the aqueous phase to retain the phase interface within the membrane pores. The design is the subject of at least two patent applications (Sakata et.al. 1986, Shimizu and Yamane 1984) in which the generic nature of the reactor is emphasised.

Cho and Schuler (1986) have applied a membrane bioreactor to a model extractive fermentation of ethanol from S. cerevisiae. Membranes were utilised to separate gas, culture, medium and extracting solvent phases, see Figure 1.7. The reactor successfully boosted ethanol yields and the generic usage of the design for product inhibition limited fermentations was suggested.

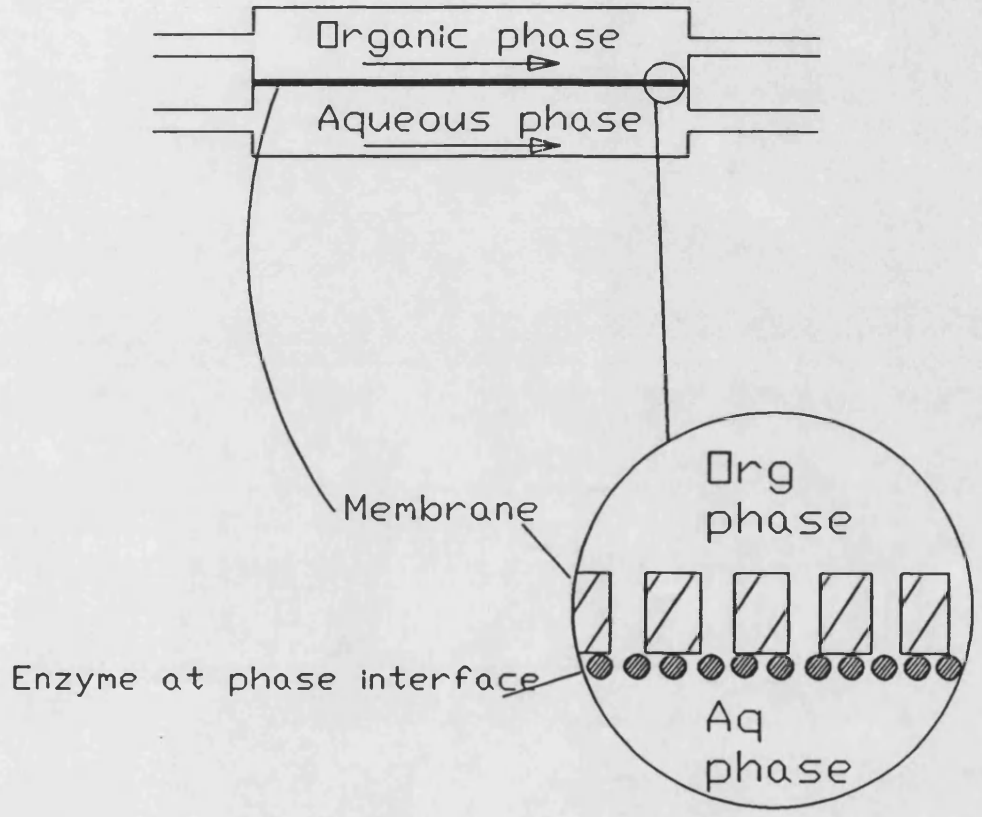


Fig 1.6 Typical Format for a Membrane Immobilised, Two-Phase Enzyme Reactor

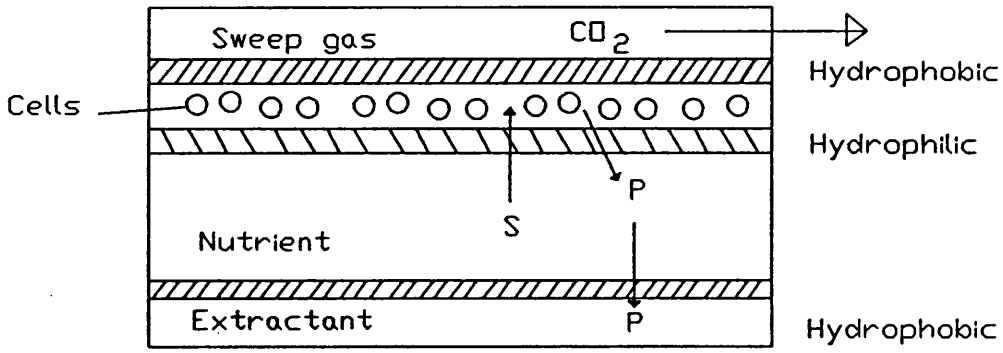


Fig 1.7 Schematic Layout of an Extractive Fermentation Membrane Reactor. From Cho T and Shuler, M. L. 1986.

1.6 SUMMARY

It is clear from the literature reviewed that aqueous-organic, two-phase bioreactions are of great potential value to a variety of chemical and biochemical based industries. Of particular value is the potential of many of these systems to replace complex or low yielding chemical syntheses with a single step reaction of high yield. The science is still in its formative years and much more research is required to elucidate the more general features critical to effective biocatalysis in organic solvents.

1.7 PROJECT OUTLINE AND AIM

Hoq et.al. (1985a, 1985b, 1986) and Shimuzi (1984) have exploited a membrane reactor for aqueous-organic, two-phase biocatalytic synthesis and hydrolysis utilising lipases. In the work reported in this thesis it was proposed to extend this approach to produce a reactor suitable for use with oxidoreductase enzymes and cofactor requiring multienzyme complexes by incorporating a coenzyme regeneration system into the aqueous phase to allow for the use of less than stoichiometric amounts of coenzyme. The proposed reactor design is shown schematically as Figure 1.8.

The aim of this work was to assess the feasibility of the novel reactor as a generic design for biotransformations where the substrate/product is poorly soluble

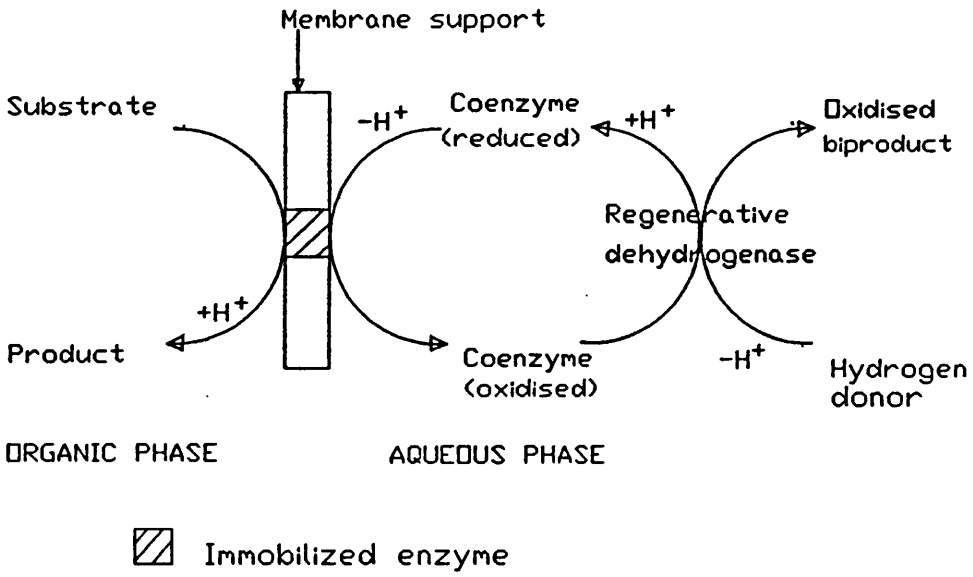


Fig 1.8 Generalised Scheme for an Oxidoreductase Catalysed Reaction in a Two-Phase Membrane Reactor.

in aqueous media and where the biocatalyst has a coenzyme requirement.

1.8 INTRODUCTION TO THE EXPERIMENTAL WORK

1.8.1 The Experimental Approach

In the experimental work techniques were developed to immobilise Horse liver alcohol dehydrogenase (HLADH) onto membranes and to monitor the resultant enzyme activity with a model substrate (octan-1-ol) in a two-phase system.

To gain insight into the kinetic behaviour of the immobilised HLADH and the factors influencing its activity, parallel experiments were performed, using both soluble enzyme in aqueous solution and membrane immobilised enzyme in a two-phase system. In this way, a comparison could be made between the intrinsic properties of the enzyme (i.e. the behaviour of soluble enzyme in aqueous solution) and the changes induced by immobilisation in a two-phase system. An extension of this work involved analysis of the physicochemical properties of the membrane support to assist understanding of the immobilisation process and factors affecting the immobilised enzyme's activity.

Further experimental work addressed the performance of coenzyme regeneration systems, utilising and extending techniques developed for the kinetic analysis of the immobilised enzyme.

1.8.2 Choice of the Model Biocatalyst and Substrate

HLADH represented an ideal choice as the biocatalyst for several reasons. Firstly, the enzyme was extremely well characterised and so, much data was available in the literature with regard to suitable methodologies and comparable results (eg. Brändén et.al. 1975).

Secondly, the substrate specificity of the enzyme was known to be broad (Winer 1958) which allowed the freedom to investigate a range of substrate activities. This was an additional benefit as many of the substrates of the enzyme were themselves hydrophobic organic solvents. Hence, using HLADH as the biocatalyst allowed variation of the concentration of the substrate in the organic phase from 0 to 100%. Finally the enzyme had been used previously in aqueous-organic, two-phase biocatalysis and so was known to be active in such systems (Martinek et.al. 1982, Larsson et.al. 1987).

A problem with the use of HLADH was the reported low stability of the enzyme (Brändén et.al. 1975). However, this problem was not immediately of major significance as the initial objective of the project was to obtain a working system not one with stable activity over long periods. A further disadvantage of the enzyme was its low specific activity (1-2 units / mg protein) this meant high activities were only possible by immobilisation of large amounts of protein. The broad substrate specificity of HLADH has the potential disadvantage that it increases the possibility of contaminating alcohols, present as solvent impurities, acting as additional

substrates. Also, the broad substrate specificity of the enzyme meant that there were only limited possibilities of performing regio-specific reactions.

Octan-1-ol was selected as a suitable model substrate for the enzyme. This compound is markedly hydrophobic (log P= 2.9, Laane et.al. 1987), can be diluted with a suitable solvent to allow variation of substrate concentration and it has been shown that the enzyme maintained a high degree of activity with the substrate. According to the literature, with longer chain more hydrophobic alcohols, eg. decan-1-ol, activity of the enzyme is reduced and although shorter chain alcohols retain high activity they were markedly less hydrophobic (Martinek et.al. 1982). The use of diols or triols etc. may have introduced complex kinetics due to a degree of regio-specificity. For these reasons octan-1-ol appeared to be the most promising model substrate.

2,2-4 trimethyl pentane (iso octane) was chosen as the organic phase solvent as it was hydrophobic in nature (log P= 4.5) and so according to Laane et.al. (1987) was unlikely to denature the enzyme.

CHAPTER 2

CHEMICALS AND MATERIALS

2.1 CHEMICALS

All laboratory reagents were of AnalaR® grade or of the highest purity readily available and were purchased from BDH Ltd, Poole, Dorset. Specialised chemicals were purchased from the following manufacturers:

Sigma Chemical Co. Ltd., Poole, Dorset.

Horse liver alcohol dehydrogenase (HLADH), quoted activity: 1-2 units / mg protein using ethanol as a substrate. Activity using octan-1-ol as a substrate has been reported as the same as that for ethanol (Sund and Thorell, 1963).

Yeast alcohol dehydrogenase (yeast ADH), quoted activity: 300-400 units / mg protein using ethanol as a substrate.

β-Nicotinamide adenine dinucleotide (NAD^+) grade III-C

Sodium dodecyl sulphate

BDH Ltd., Poole, Dorset.

2,2,4-Trimethyl pentane HiPerSolv®

2.2 MATERIALS

Specialist materials were obtained from the following manufacturers:

Millipore Ltd., Watford, Herts.

0.1, 0.22, 0.45 and 0.65 μm polyvinylidene difluoride (PVDF), Minitan® membrane cassettes

Portmere Rubber Ltd., Southampton, Hampshire.

1 mm Nitrile rubber sheet type p 559.

BDH Ltd., Poole, Dorset.

Viton, Masterflex tubing

Chromatography Services Ltd., Wirral, Merseyside.

5 ft. glass gas liquid chromatography (GLC) column prepacked with Porapak®P

CIL Group, Sussex.

Analogue to digital, digital to analogue, Input/output PC super card fitted to an Opus III, IBM compatible computer)

Dr. J. Hubble, School of Chemical Engineering, University of Bath

In-house enzyme kinetics analysis program using the direct linear plot method (Eisenthal and Cornish-Bowden 1974).

CHAPTER 3

DEVELOPMENT OF A SMALL-SCALE, TWO-PHASE ENZYME REACTOR

3.1 OUTLINE OF THE REACTOR

A small glass reactor was constructed for the purpose of studying two-phase reactions with immobilised HLADH (see Figure 3.1). The reactor was constructed in two parts to allow the formation of two compartments separated by the membrane. During reactor operation, the organic phase was placed in the bottom compartment and the aqueous phase in the top. The membrane was sandwiched between the flanges separating the phases. The membrane area was 11.95 cm^2.

3.2 PRELIMINARY EXPERIMENTS AND THE DEVELOPMENT OF A STANDARDISED METHOD OF ENZYME IMMOBILISATION

The immobilisation method decided upon was adsorption as it is a straight forward means of enzyme immobilisation. Additionally, the technique is mild and so the degree of activity retained can often be higher than that obtained using chemical attachment (Trevan 1980).

One of the first tasks was to demonstrate immobilised enzyme activity in a

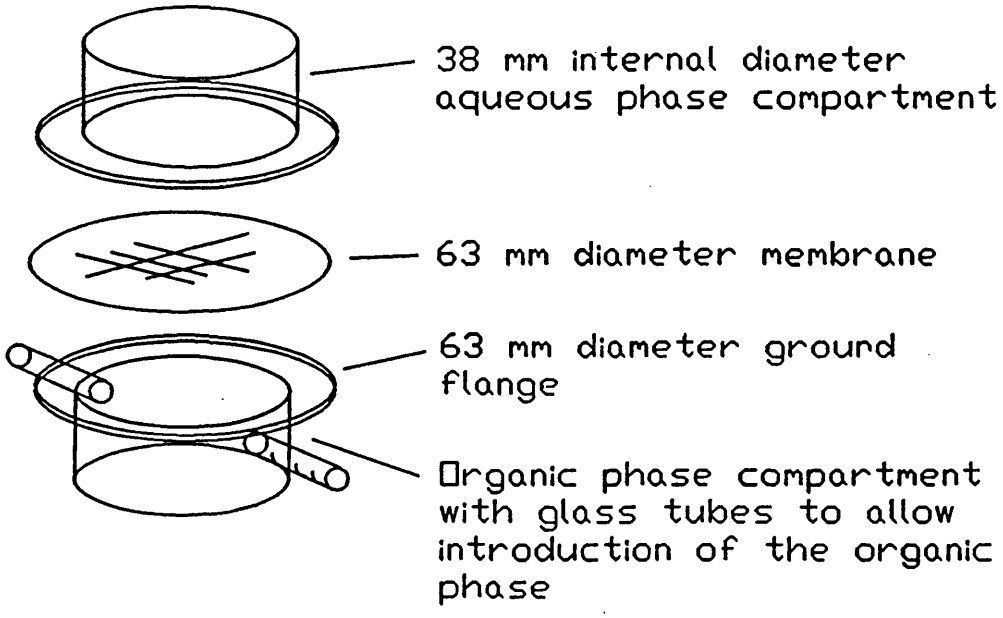


Fig 3.1. The Small Glass Reactor

two-phase system to allow assessment of the feasibility of the reactor concept and to ensure the enzyme did not desorb under reaction conditions.

The following experimental protocol was used to assess both the stability of enzyme immobilisation and the activity of the immobilised material. A 63 mm diameter membrane circle was cut from a 0.22 μm Millipore Minitan® membrane cassette and placed in 0.1 M, pH 9.5 NaOH-glycine buffer to equilibrate for 15 minutes. The membrane was blotted dry and placed in 10 ml of the same buffer containing 1 mg/ml HLADH. After 30 minutes the membrane was removed, blotted dry and washed in 100 ml buffer for 5 minutes. This process was then repeated. The washing process was designed to ensure that no soluble enzyme was carried over from the immobilisation process.

The membrane was then placed in the reactor with its "permeate" side facing the aqueous phase. 18 ml of 2,2,4-trimethyl pentane containing 20% (v/v) octan-1-ol was introduced into the lower compartment. Care was taken not to introduce air bubbles into the solvent which could be trapped against the membrane and modify its effective area. The reactor was placed on a magnetic stirrer and an overhead stirrer positioned in the aqueous phase compartment. 20 ml of buffer was then introduced into the aqueous phase compartment and mixing of both the aqueous and organic phases was begun. 20 mg of solid NAD^+ was then added to the aqueous phase compartment to start the reaction. Periodically, 1 ml samples of the aqueous phase were removed. The absorbance of each sample was read at 340 nm against a buffer blank using a Cecil CE 588 spectrophotometer to determine the extent of NADH

production in the reactor (the extinction coefficient of NADH was taken to be 6220 $l.mol^{-1}.cm^{-1}$). To each sample was added 150 μl of a solution of 104.5 mg/l octan-1-ol in 0.1 M, pH 9.5 NaOH-glycine buffer. Samples were then left in the cuvette holder until a fresh sample was taken, so it was possible to monitor the residual reaction rate attributable to desorbed enzyme. Samples were not returned to the reactor.

This procedure allowed the effectiveness of the immobilisation procedure to be assessed by identifying the contribution of both soluble and immobilised enzyme to the observed reaction rate. The results are shown in Figures 3.2 and 3.3.

The increase in NADH concentration over time suggested the immobilised enzyme was functioning catalytically. However, the change in NADH concentration of the aqueous phase samples, standing in cuvettes in the spectrophotometer, suggested that some soluble enzyme was present. From these results, for the reaction rate observed in samples taken from the reactor the amount of soluble enzyme present could be estimated.

Enzyme activity obtained for HLADH using octan-1-ol as a substrate is identical to that obtained using ethanol (Sund and Theorell, 1963); approximately 1.5 μmoles NADH/min/mg enzyme. Assuming no limitation to the rate of reaction ie. no product inhibition and no substrate (octan-1-ol) limitation, then for a mean rate of change in absorbance of samples standing in the spectrophotometer of 0.0015 A/minute, the amount of enzyme in a 1 ml sample was found to be approximately 0.3 μg. Relating this to the starting volume of 20 ml indicates approximately 6 μg of

enzyme was released from the membrane. It was doubted that any carry over of soluble enzyme from the membrane washing procedure was responsible for this effect as washing and blotting of the membrane were thorough.

A possible explanation for the enzyme desorption under run conditions was the presence of NAD^+ in the buffer used. In this experiment the immobilised enzyme was not subject to a wash in buffer containing NAD^+ but, NAD^+ was present in the aqueous phase during reaction. The inclusion of NAD^+ into the aqueous phase would have produced an increase in the ionic strength of the solution relative to the immobilisation solutions. Desorption of proteins in the presence of increased ionic strengths has previously been observed (Trevan 1980) and may have occurred in this case. Also NAD^+ would have been available to bind to its catalytic site on the enzyme which could have caused enzyme desorption in itself. It was considered probable that presence of NAD^+ was responsible for desorption of active enzyme from the membrane and presence of active soluble enzyme lead to the subsequent changes in absorbance of samples removed from the reactor and monitored in the spectrophotometer. Contribution to enzyme desorption by NADH produced in the reaction was considered unlikely. As can be seen from Figure 3.3 the accumulation of soluble enzyme was rapid and did not increase over time as might have been expected if accumulated NADH was leading to desorption.

In all further experiments membranes were washed for at least 30 minutes prior to use in 10 ml, 0.1 M pH 9.5 NaOH-glycine buffer containing 1 mg/ml NAD^+. This ensured that the immobilised enzyme was exposed to a solution of

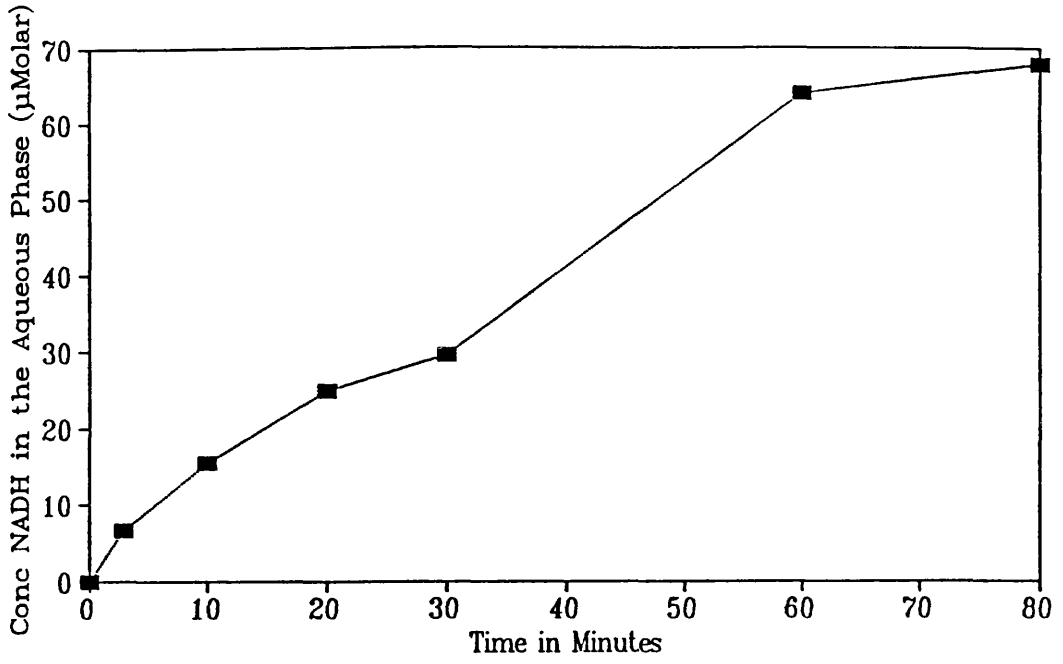


Fig 3.2. Preliminary Experiment in the Small, Glass Reactor.
Conditions: aqueous phase– 20mg NAD in 20 ml 0.1 M, pH 9.5 NaOH–glycine buffer; organic phase– 18 ml 2, 2, 4– trimethyl pentane containing 20% (v/v) octan–1–ol Temperature– 17°C.

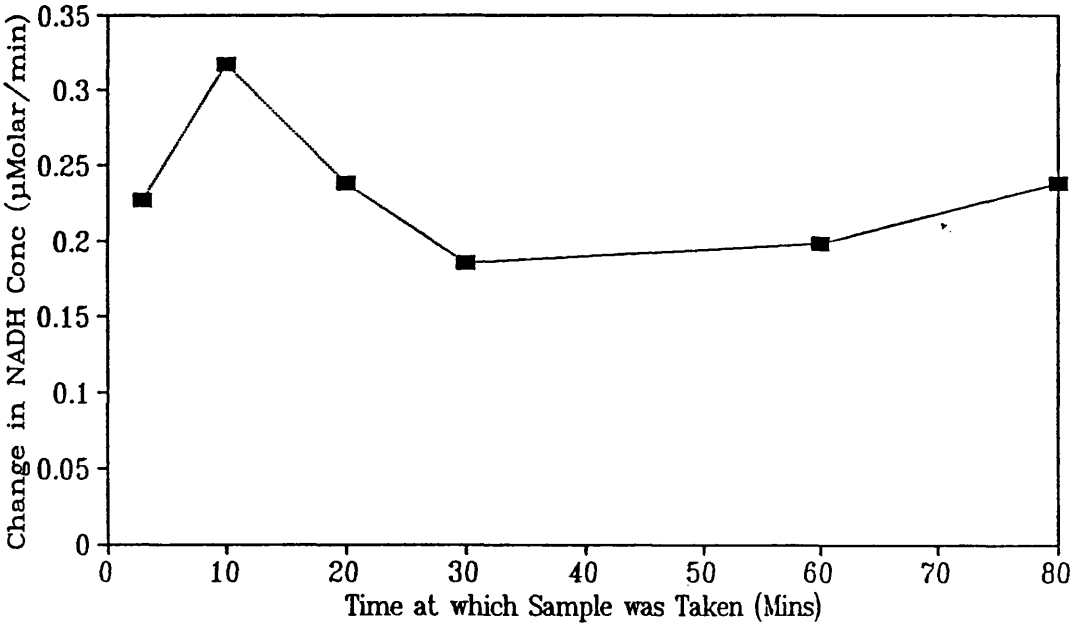


Fig 3.3. Reaction Rates Produced by Aqueous Phase Samples Removed from the Reactor.
1 ml samples of the aqueous phase were removed from the reactor and assayed at 340 nm, any change in absorbance was monitored by leaving the sample in the spectrophotometer.

equivalent chemical composition to the aqueous reaction phase before its introduction to the reactor such that any desorption of enzyme due to the presence of NAD^+ did not interfere with subsequent monitoring of the reaction kinetics. Following implementation of this technique no other samples of aqueous phases tested showed increases in absorbance at 340 nm.

Following these experiments, a standard immobilisation procedure and a standard reactor configuration were adopted.

3.3 STANDARDISED METHODS OF MEMBRANE PREPARATION, ENZYME IMMOBILISATION AND ENZYME RECOVERY

The method used was designed to measure the amount of enzyme immobilised by its recovery from the membrane using a detergent (SDS) in alkaline solution. A similar method had been used previously (Suki, Fane and Fell 1984) and was considered to remove all adsorbed protein from membranes. The other possible approach was to monitor changes in the amount of soluble enzyme during the immobilisation and washing stages to obtain the amount of enzyme immobilised via a mass balance. This option represented a less direct technique which would have required the analysis of far more samples for each assay and was therefore potentially subject to larger errors. Additionally, as the total recovery method utilised a detergent to remove the enzyme from the membrane it meant that the membrane was also partially cleaned and regenerated by this procedure.

3.3.1 Preparation of Fresh Membranes and Regeneration of used Membranes

The following procedure was adopted to provide a reproducible membrane cleaning protocol to remove enzyme from the membrane and regenerate it to its virgin state. This allowed reuse of the same membrane where an enzyme kinetic experiment was conducted over several days. Hence use of the procedure avoided possible variation of results caused by the use of several membranes within a single experiment.

63 mm diameter circles of PVDF (polyvinylidene difluoride) membranes were cut from Millipore Minitan® membrane cassettes. Circles were then washed overnight in 10 cm^3, 5% (w/v) Sodium Dodecyl Sulphate (SDS) in 0.5 M NaOH at 30°C (this temperature was required to prevent precipitation of the SDS from solution). The membranes were then washed in at least 250 ml distilled water and placed in 3% (w/v) Tergazyme® solution for at least 1 hour. Following washing in 4 changes of distilled water the membranes were placed in 0.1 M, pH 9.5 NaOH-glycine buffer for at least 15 minutes before use to equilibrate.

3.3.2 Enzyme Immobilisation

The pre-equilibrated membranes were blotted dry and placed in 10 ml, 0.1 M, pH 9.5 NaOH-glycine buffer containing 1 mg/ml HLADH for 30 minutes. Membranes were then blotted dry and subject to 2 washes in 10 ml of the same buffer

(without enzyme present) for 15 minutes each. Finally, the membranes were blotted dry and placed in 10 ml of the same buffer containing 1 mg/ml NAD^+ for at least 30 minutes. The membranes were removed and blotted dry before use.

3.3.3 Determination of Adsorbed Protein

After a series of experiments were complete the membranes were blotted dry and placed in 5% (w/v) SDS in 0.5 M NaOH overnight at 30°C. The following day a sample of the solution, containing solubilised protein, was taken for assay (Suki, Fane and Fell 1984). The membrane was then placed in 3% (w/v) Tergazyme® and from there on treated the same as a fresh membrane (as outlined above in Section 3.3.1).

The collected sample was then assayed for protein using the Lowry method modified for proteins solubilised in SDS (Hess, Lees and Derr 1978). The method is outlined in the reference except that in this application it was found necessary to microcentrifuge samples before spectrophotometric determinations could be carried out with any accuracy. The standard protein used was HLADH. A typical standard curve obtained using the method is included as Figure 3.4. It can be seen that the response of the assay was linear over the HLADH concentration range used.

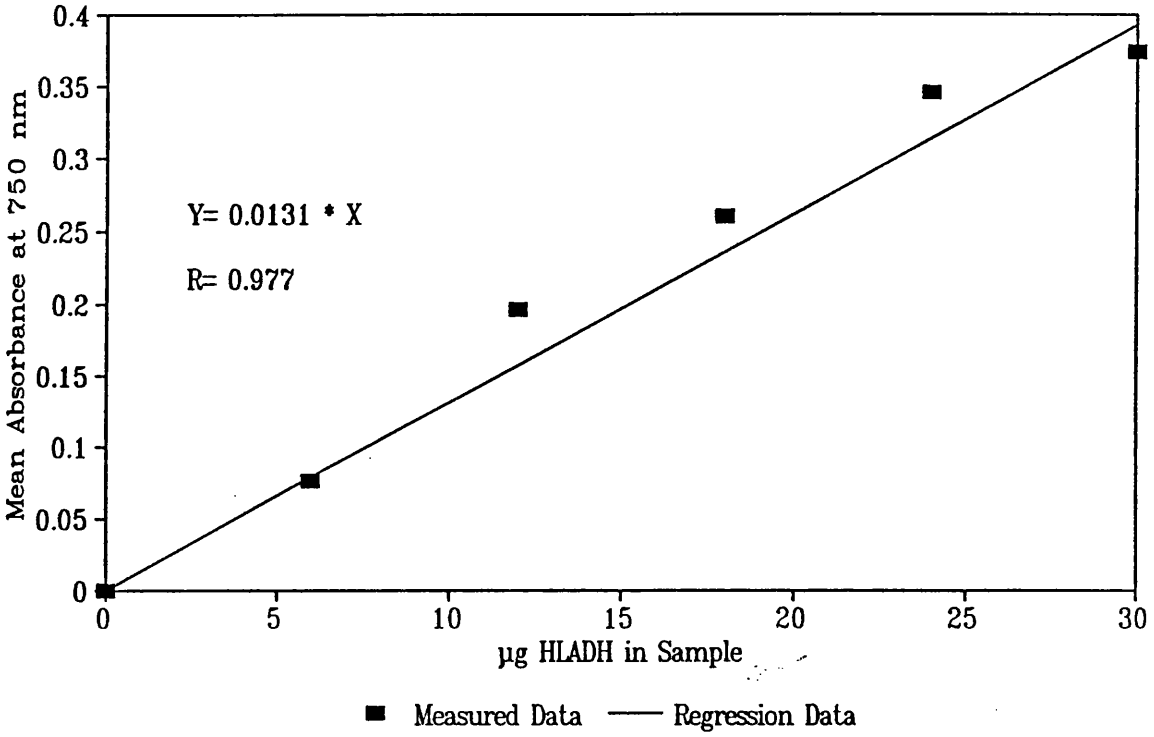


Fig. 3.4. A Typical Standard Curve Obtained from the SDS Modified Lowry Assay.

3.3.4 Validation and Analysis of the Protein Recovery Method

To confirm that the estimates of adsorbed protein obtained using the technique above were realistic, a mass balance was carried out on the immobilisation procedure to provide a comparative measure of the amount of enzyme adsorbed to a membrane.

A 0.22μm membrane was taken, prepared and HLADH immobilised per Sections 3.3.1. and 3.3.2. The initial mass of HLADH used was accurately measured and samples of the load and wash buffers were taken after each step of the immobilisation procedure was complete. The protein content of these samples was estimated using the same Lowry protein assay utilised in Section 3.3.3 however, the standards used were of HLADH in 0.1 M, pH 9.5 NaOH buffer.

Following immobilisation, the adsorbed enzyme was recovered and assayed using the techniques described in Section 3.3.3. These results allowed calculation of the total amount of enzyme immobilised and in solution at each step of the process. The comparison of the mass balance and SDS wash estimates of adsorbed enzyme is shown below.

Mass Balance:

10.50 mg.....Originally added HLADH to 10 ml buffer

9.30 mg.....Recovered after the loading period

0.34 mg.....Recovered from the wash samples

9.64 mg Total recovered HLADH

Therefore, 10.50- 9.64= 0.86 mg HLADH was potentially bound to the membrane.

SDS Wash:

0.60 mg HLADH was recovered.

Errors for the protein assay procedures were approximately 5-10% for the SDS wash method and 20-40% for the mass balance method (ie. four individual protein assays). The relative high random error margin for the mass balance technique and errors due to losses of enzyme during blotting of the membrane in between washes were probably responsible for the 30% discrepancy in the two results. It was also possible that less than 100% of the adsorbed enzyme was released upon washing in SDS due to irreversible adsorption of a proportion of the enzyme to the membrane. So the SDS wash technique may well have underestimated the amount of protein present on the membrane. Even with this limitation the technique was still capable of providing internally consistent and comparative estimates of the amount of enzyme adsorbed. In summary, it was considered the SDS wash technique provided acceptably reproducible results.

Table 3.1 shows some of the protein recovery results obtained over the course of the project and relates them to the reaction conditions and the reaction rates obtained. Overall the protein recoveries showed a percentage variance (sample variance divided by the sample mean, multiplied by 100) of 30.5% and a percentage variance of the reaction rates of 12.2%. Obviously there was a general relationship

Membrane Number	Protein Recovered(μg /cm^2)	Reaction Conditions	Reaction Rate (μMol NADH/min)	Specific Reaction Rate(μMol NADH/min/ mg HLADH)
1	19.3	Organic Phase: 10% Octan-1-ol Aqueous Phase: 1mg/ml NAD^+	0.007	0.030
1	13.4	"	0.005	0.029
1	15.1	"	0.005	0.030
1	10.9	Organic Phase: 1% Octan-1-ol Aqueous Phase: 1mg/ml NAD^+	0.003	0.025
2	19.2	"	0.007	0.030
3	23.4	"	0.006	0.022
4	24.3	"	0.008	0.027
5	11.3	"	0.003	0.023

Table 3.1 **Summary of Enzyme Recoveries from a Range of Experiments Performed Throughout the Project**

The aqueous phase consisted of 0.1M, pH 9.5 NaOH-glycine buffer with NAD^+ added as detailed. The organic phase consisted of 2,2,4-trimethyl pentane with octan-1-ol added as detailed. All membranes were loaded under the same conditions as described in Section 3.3.2.

where reaction rates increased with protein recoveries, however as can be seen from the comparison of % variance this relationship was not directly proportional. This suggested not all the (active) enzyme was freely accessible for reaction with the substrates: if the enzyme was freely accessible then these two values should have been consistent. This point will be further addressed in the discussion (Chapter 8).

Variation of the amount of protein recovered was too large to be accounted for by errors introduced by the assay (5-10%) and was probably caused by: variation between batches of enzyme: the history of the individual membrane samples (eg. recovery losses introduced by irreversible adsorption probably changed over time) and: comparison of results between membranes (samples of a single membrane sheet have been observed to possess large variation of their physical properties (Pritchard 1990) which suggests variation of adsorption capacity between membranes).

3.4 DEVELOPMENT OF A SMALL-SCALE RIG

Following the establishment of suitable supportive analytical methods it was necessary to develop techniques to investigate the kinetics of the immobilised enzyme. The experimental techniques used in the preliminary experiment with the small glass reactor were refined and investigated for possible sources of experimental error.

A rig was designed, based around the small glass reactor which allowed continuous monitoring of the aqueous phase absorbance at 340 nm. This facility made

it possible to carry out initial rate determinations with the immobilised enzyme. The rig design is shown schematically as Figure 3.5.

3.4.1 Development of Data Logging Software

To facilitate the recording of instrument outputs and the rapid manipulation of experimental data an Opus III, IBM compatible computer was purchased and fitted with a proprietary analogue to digital converter. This device allowed the recording of up to 8 separate instrument outputs simultaneously. Additionally, the input/output device gave the facility to control laboratory equipment eg. a pH stat. Such a facility was envisaged as a possible requirement.

A BASIC program was written, using Turbo BASIC (Borland, Twyford, Berks.), to periodically log analogue outputs from laboratory instruments and to display the data on the computer monitor screen as a continually updated "chart recorder" image. The data could also be logged to disk if required. In this way, the computer provided the ability to monitor a process much as though a traditional chart recorder. Moreover, as the data was stored directly in digital form, post-mortem data analysis and interpretation was greatly facilitated. A flow diagram of the program is shown as Figure 3.6. The program listing is included as Appendix 1.

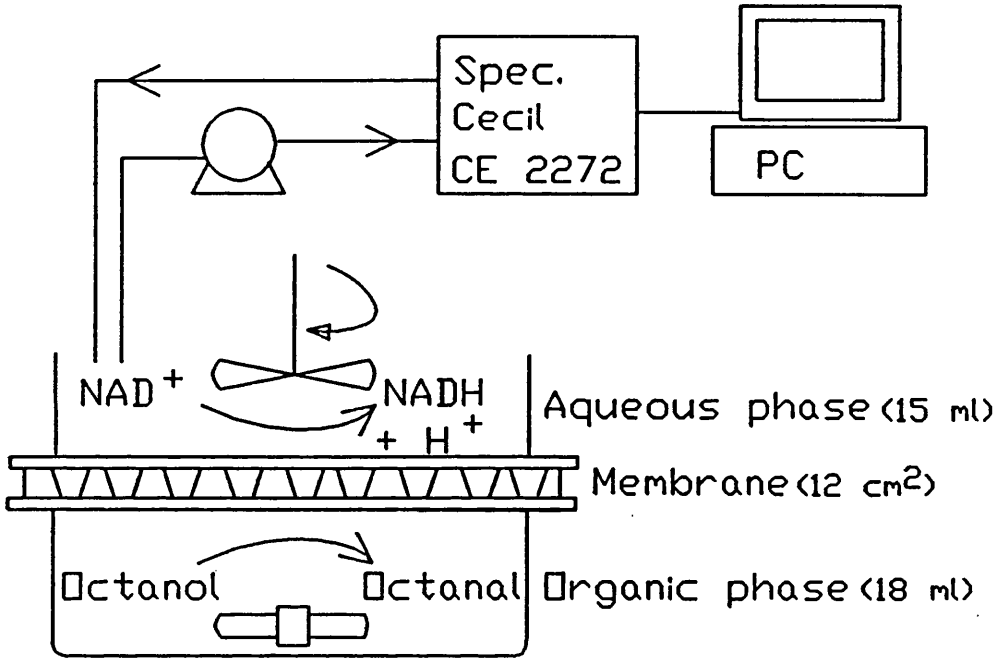
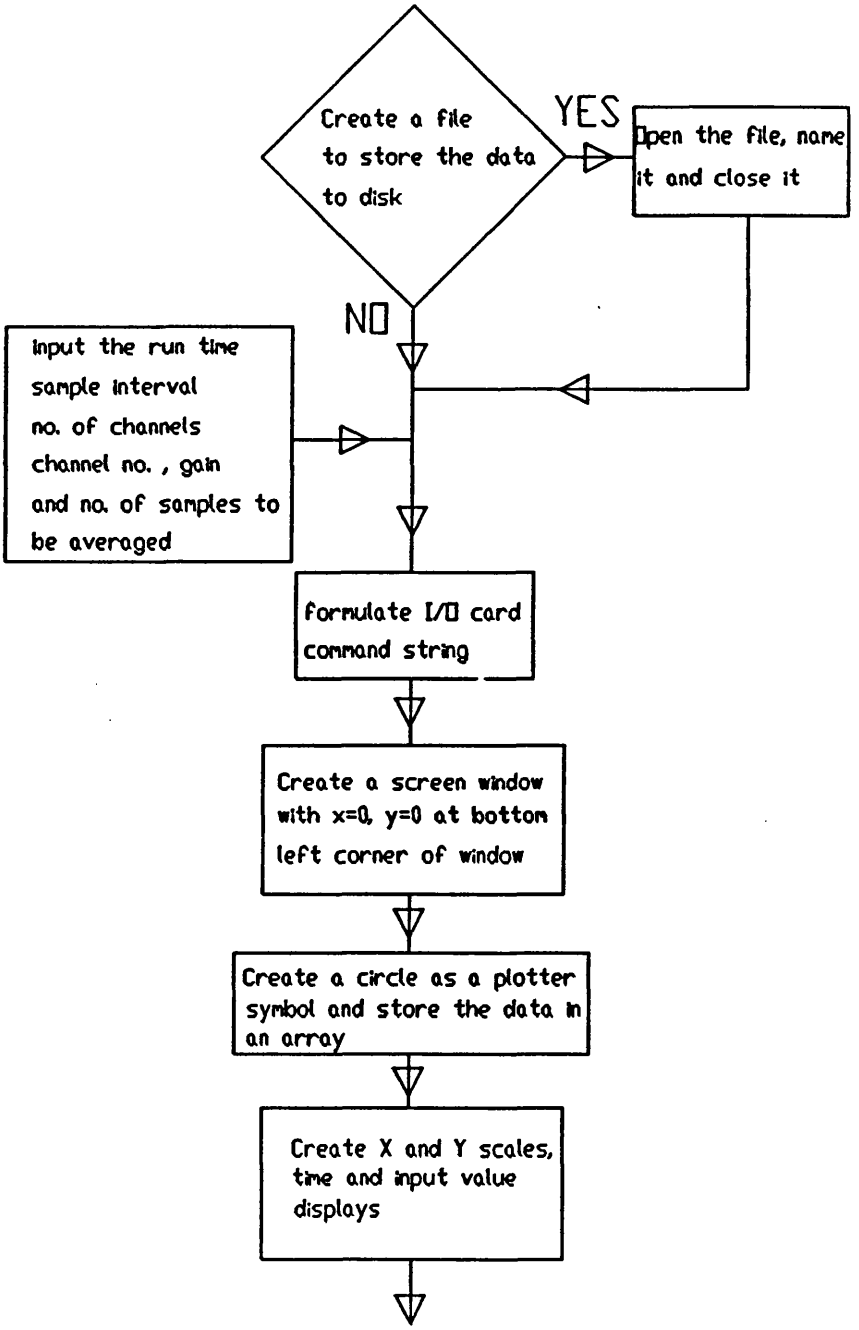


Fig 3.5 The Overall Rig Layout

Fig 3.6 A Flow Diagram of the Data Logging Program



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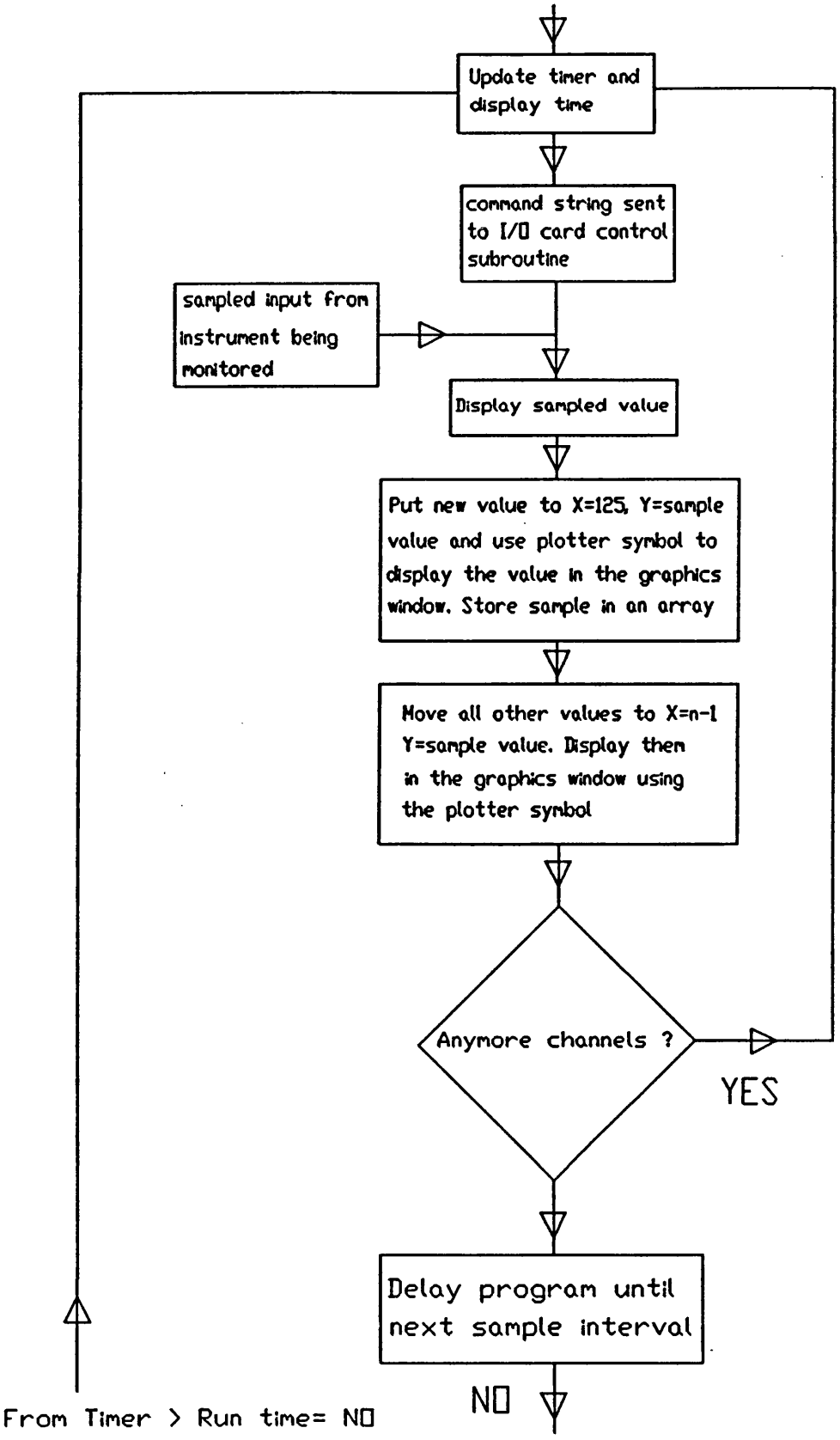

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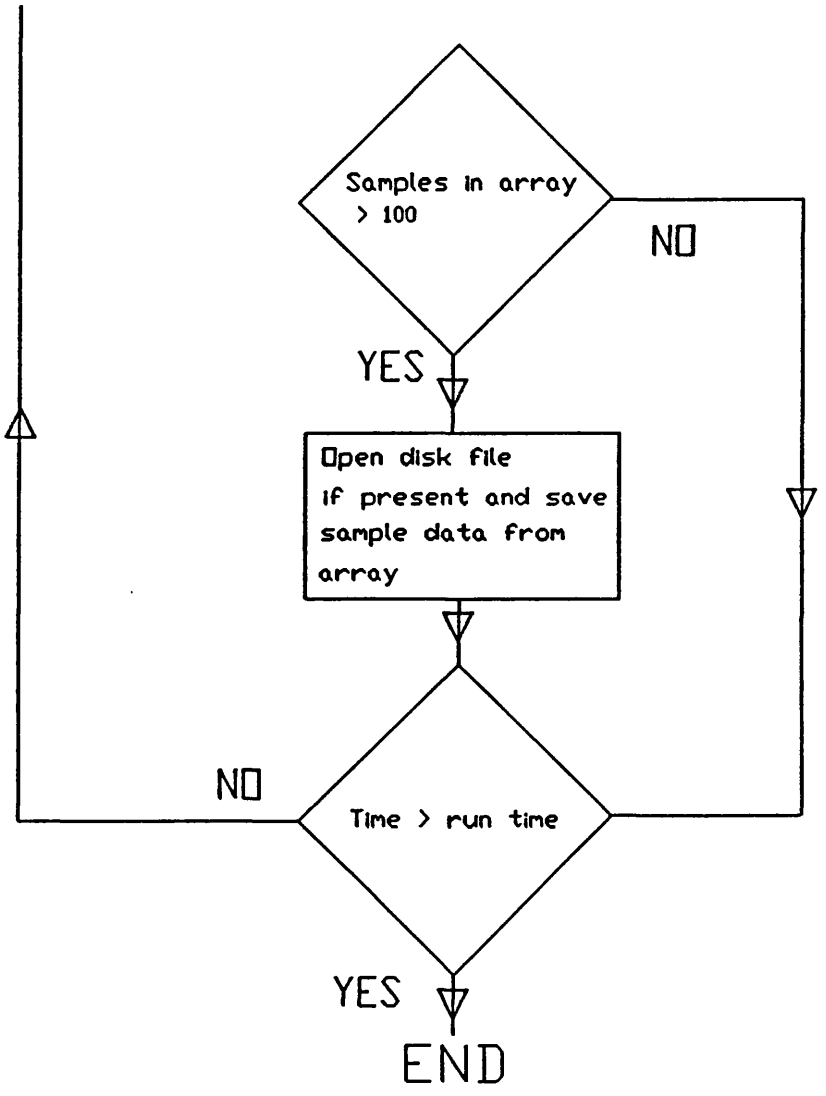

Samples in array
> 100
NO
YES
Open disk file
if present and save
sample data from
array
NO
Time > run time
YES
END

3.4.2 Calibration of the Spectrophotometer Input

A full scale deflection of 2 absorbance units was selected on the spectrophotometer and the absorbance adjusted to 2 units using the back off control. Approximately 50 output samples were then logged over a minute. The back off control was then randomly adjusted and the spectrophotometer reset to 2 units. The output was logged again. The procedure was then repeated once more to give in total 3 sets of output data at 3 independent estimations of the correct absorbance value. These results were then averaged to give a reliable estimate of the true value. The whole procedure was repeated at 1.5, 1.0, 0.5 and 0 absorbance units. This series of data was then used to produce a straight line regression correlation of the spectrophotometer output against absorbance. This correlation was used to convert logged data into absorbance units. Similar correlations were derived for the 0.5 and 0.2 scales on the spectrophotometer. The calibration was periodically checked for consistency. A sample standard curve obtained is shown as Figure 3.7. As can be seen from Figure 3.7 the system produced an accurate, linearly proportional response.

3.4.3 Commissioning of the Small Glass Reactor

To provide reliable reaction rate estimates it was necessary to ensure that the increase in absorbance at 340 nm was solely produced by reduction of NAD^+ to NADH and was not an experimental artifact. To this end a series of control experiments were carried out.

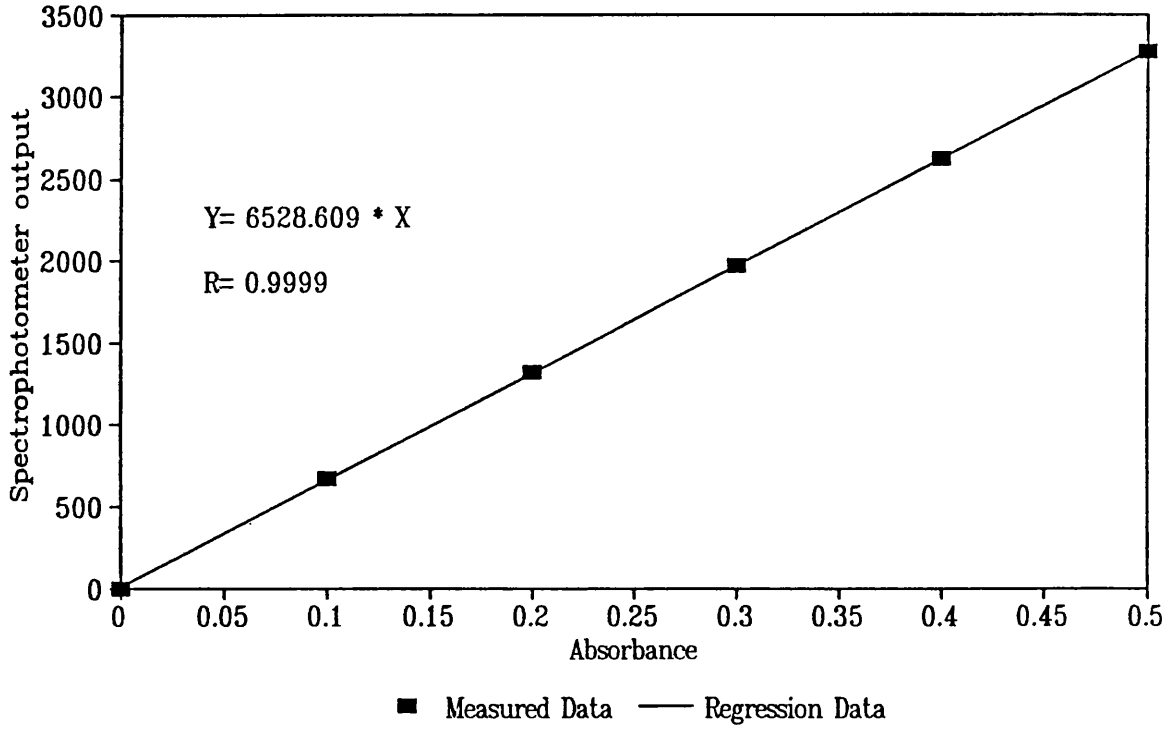


Fig. 3.7. A Typical Calibration Curve for the Spectrophotometer using the 0.5 Absorbance Scale. The spectrophotometer output is in an arbitary scale which represents its voltage output from 0 to 10 mV (ie. 0 to 3500).

The choice of materials was found to have a marked effect on the results obtained. In a preliminary experiment aimed at using a Millipore Minitan® microfiltration unit as an immobilised enzyme reactor (see Chapter 8 for further details of experiments using this equipment) it was noticed that reactive species were being leached from the silicone tubing used to connect the aqueous and organic phase vessels to the reactor. During the experiment both aqueous and organic phases of the Minitan® unit were continuously recirculated as shown in Figure 3.8. The aqueous phase consisted of 0.1M, pH 9.5 NaOH-glycine buffer only. The organic phase consisted of 1% octan-1-ol in 2,2,4-trimethyl pentane. No enzyme was immobilised on the membrane.

Upon starting recirculation of the phases samples of the aqueous phase were taken and enzymatically assayed (using HLADH) for octan-1-ol concentration. The enzymic assay technique is described fully in Section 6.5. It was expected that the octan-1-ol concentration of the aqueous phase would increase over time and reach a constant value dependant on the partition coefficient of the system for octan-1-ol. The actual result obtained is shown as Figure 3.9. As can be seen, after approximately 40 minutes the system appeared to be approaching equilibrium. However, after this period the apparent amount of octan-1-ol present continued to increase with time. This effect which was reproducible, was thought to arise from substrates for HLADH being leached from materials used in construction of the rig. A number of materials were isolated as suspect: silicone grease, silicone tubing and rubber. These materials were investigated further.

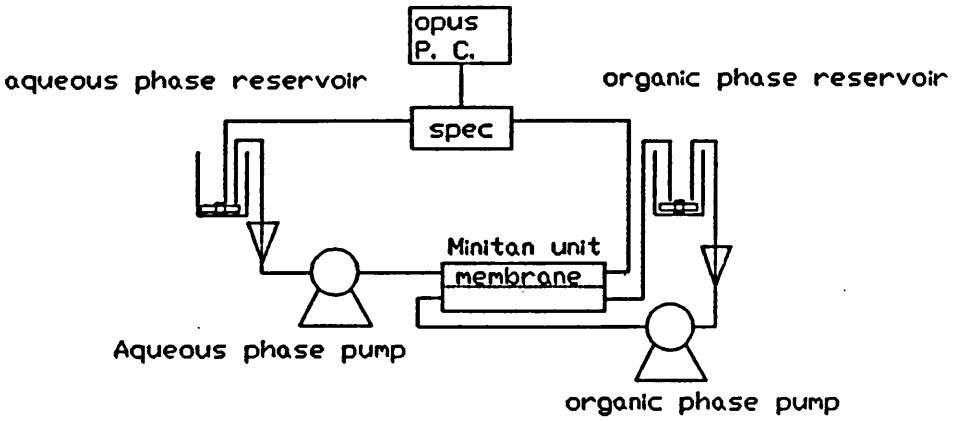


Fig 3.8 Schematic Diagram of the Minitan Reactor
Showing Continuous Recircultation of Both the Phases

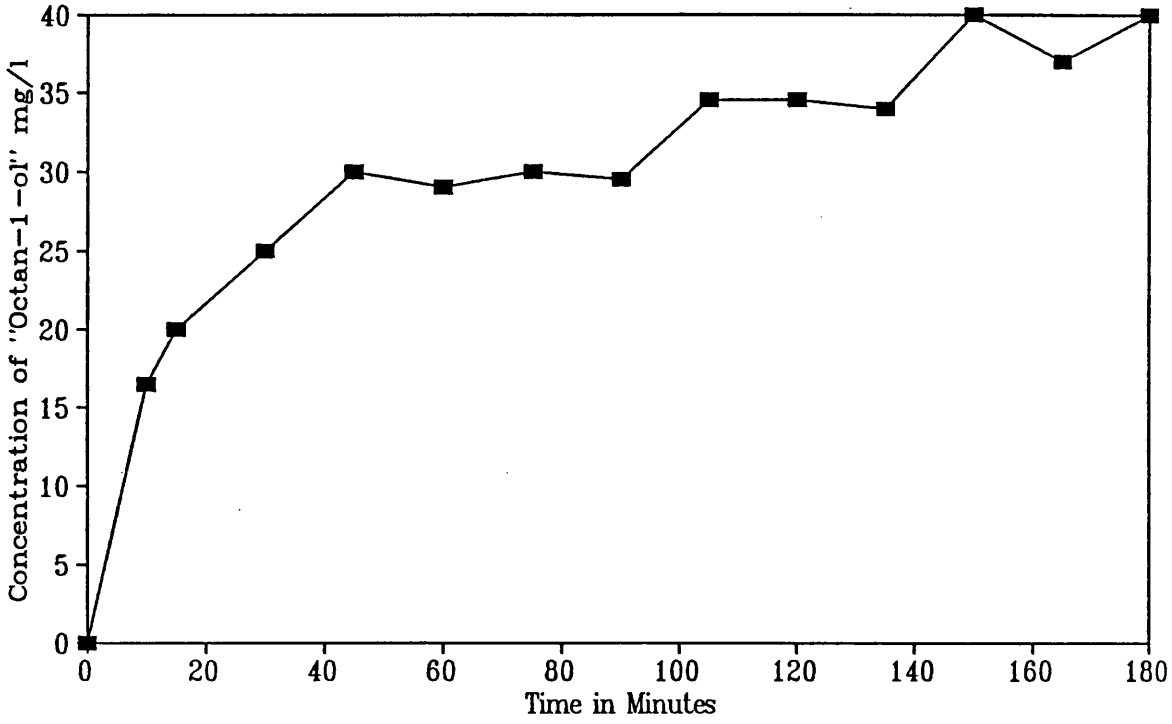


Fig 3.9. Apparent Increase in Octan-1-ol Concentration of the Aqueous Phase in the Minitan Rig. Both phases recirulated, octan-1-ol assayed enzymatically

Approximately 20ml, 0.1M, pH 9.5 NaOH-glycine buffer was stirred in a glass beaker and 5 mg HLADH and 20 mg NAD^+ added. Finally a sample of the suspect material was added and the increase in absorbance at 340 nm of 3 ml samples monitored. Only silicone tubing produced a measurable change in absorbance of 0.047 A/minute. It was concluded that the enzyme was catalytically active with materials (presumably alcohols) released from the silicone tubing into solution. Viton rubber tubing was tested and this produced no reaction and so this material and glass tubing replaced silicone tubing in all future experiments.

Next the effect on absorbance at 340 nm of octan-1-ol and 2,2,4-trimethyl pentane partitioning into (and accumulating in) the aqueous phase was investigated. The small glass reactor was arranged as shown in Figure 3.5 and 15 ml, 1% octan-1-ol in 2,2,4-trimethyl pentane introduced into the organic phase compartment. Then 15 ml of 0.1M, pH 9.5 NaOH-glycine buffer was introduced into the aqueous phase compartment and stirring of both phases, recirculation of the aqueous phase and data logging begun. Over a 30 minute period no increase in absorbance occurred and it was concluded that partitioning of the organic solvent and substrate into the aqueous phase did not significantly effect its absorbance at 340 nm.

Finally, the quality of the 2,2,4-trimethyl pentane was found to effect results. In early experiments using AnaLaR® grade solvent reaction rates were found to be relatively insensitive to reduction of the amount of octan-1-ol incorporated into the organic phase at low octan-1-ol concentrations (0.01% or less). Upon use of a higher quality grade of 2,2,4-trimethyl pentane (HiPerSolv® grade) this effect vanished and

changes in reaction rate were observed with changes of octan-1-ol concentration. The inference is that the lower quality solvent contained trace amounts of alcohols which were acting as substrates for the immobilised enzyme.

Following implementation of the controls outlined in the above paragraphs initial reaction rate results obtained using the system were very close to linear. A typical output logged during such an experiment is shown as Figure 3.10. The conditions for this experiment were; organic phase- 18 ml 2,2,4- trimethyl pentane containing 1% octan-1-ol; aqueous phase- 15 ml 0.1 M, pH 9.5 NaOH-glycine buffer containing 1 mg/ml NAD^+. The graph shows a approximately linear increase in absorbance at 340nm with time indicating initial rate determinations made over such a period were valid. Further, as less than 1% of the starting concentrations of both substrates were converted to product over this period so their concentrations were approximately constant. So minimal deviations from the initial concentrations allowed measurement of an almost linear fraction of the whole progress curve which minimised the errors inherent in obtaining an initial rate.

3.4.4 Development of Data Analysis Techniques

The computer link allowed raw experimental results to be fed directly into computer files. Data was analyzed and displayed graphically using a Lotus 1-2-3 spreadsheet, this made repetitive calculations straightforward. Even so the sample rate of the data logger was typically set to provide around 100 measurements over an

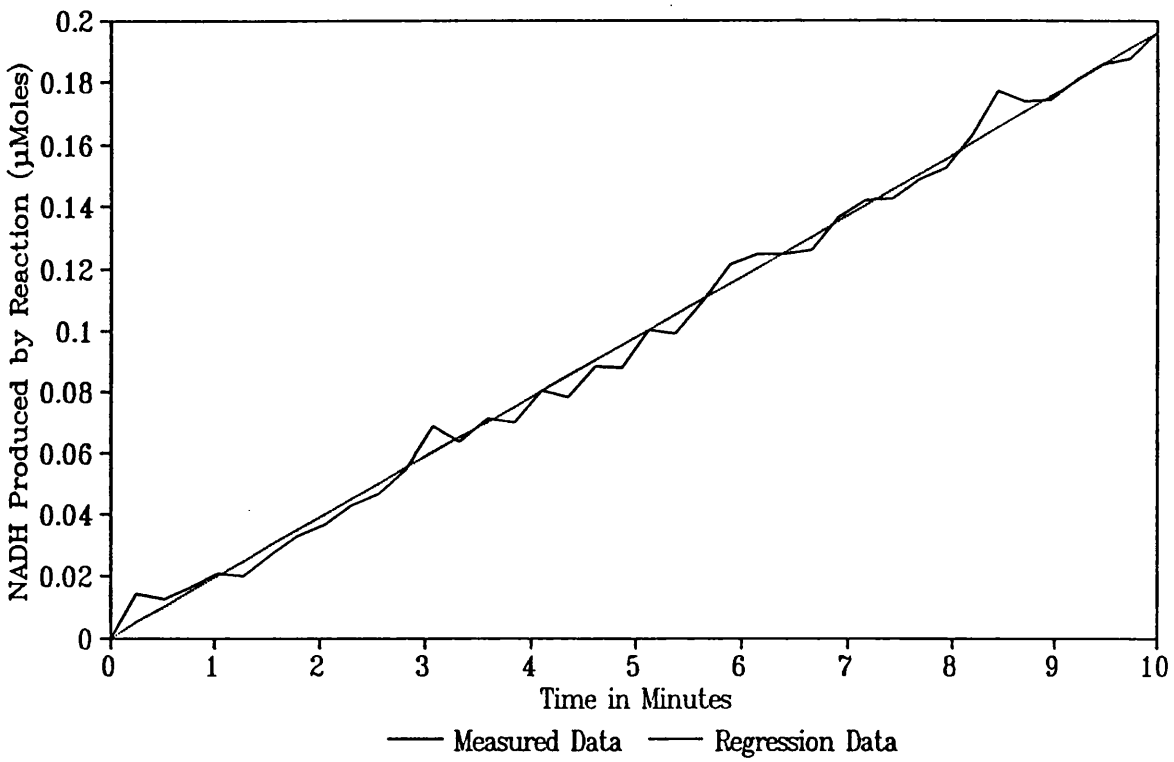


Fig 3.10. Change in Absorbance of the Aqueous Phase as Logged by the PC Showing the Linear Nature of the Rate Curve Obtained

experimental period to prevent the volume of results from becoming unwieldy.

Occasionally catchment of an air bubble in the spectrophotometer flow cell produced a non linear increase in absorbance. To avoid the inadvertent interpretation of such results as genuine the data were analyzed using the linear regression facility of Lotus 1-2-3. Data failing to produce time course profiles with linear correlation coefficients of 0.95 or better were rejected. Typically, the period between 20 seconds and 4 minutes was used for result analysis to avoid the inclusion of extremely high values introduced by the presence of an empty flow cell in the first few or last few seconds of an experiment.

Finally, data used in the calculation of kinetic constants etc. were the regression values. Thus, the regression analysis also functioned as a data smoothing facility, damping noise errors present in the results.

3.4.5 Determination of Adsorbed Enzyme Stability Over the Time Period Required for a Set of Initial Rate Experiments

This experiment was conducted as a control to ensure that decay of enzyme activity was not an influence on results obtained through conducting a series of consecutive experiments on the same immobilised enzyme preparation.

HLADH stability was determined over 9, 15 minute runs (approximately 5

hours total time) at 20°C. This represented the maximum time course of a series of runs used to determine the initial rate kinetics of the immobilised enzyme.

HLADH was immobilised to a 0.22 μm membrane as described in Section 3. The reactor was loaded with: 15 ml, 0.1 M, pH 9.5 NaOH-glycine buffer containing 1 mg/ml NAD^+ (aqueous phase) and; 18 ml 2,2,4-trimethyl pentane containing 1% (v/v) octan-1-ol (organic phase). The aqueous and organic phase stirrer speeds were set at 350 and 330 R.P.M. respectively. The increase in absorbance at 340 nm was monitored for 15 minutes. The top and bottom sections of the reactor were then rinsed with buffer and 2,2,4-trimethyl pentane, respectively. The procedure was then repeated a further 8 times.

The results obtained are displayed as Figure 3.11. The results were expressed with reference to the aqueous phase volume. The mean activity value was 0.007 μMoles NADH/min and the standard deviation was 0.0004 μMoles NADH/min which statistically indicted the consistency of the results and therefore the operational stability of the immobilised enzyme over this period.

3.4.6 Effects of Stirrer Speed on the Initial Reaction Rate

It was necessary to investigate the effects of the aqueous and organic phase stirrer speeds on observed reaction rates, to ensure the reaction was not operated under conditions of external mass transfer limitation. Under such conditions reactions

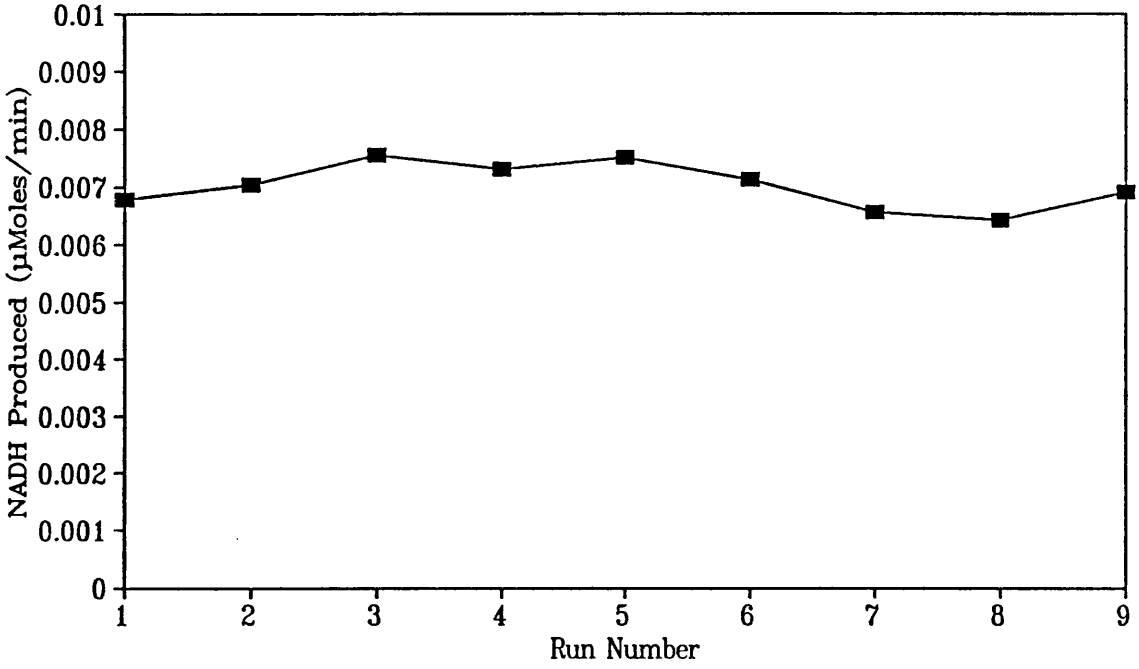


Fig. 3.11. A Series of Repetitive Runs in the Small Two-Phase Reactor. Conditions: Aqueous phase- 15 ml, pH 9.5, 0.1 M NaOH-glycine buffer containing 1mg/ml NAD; Organic phase- 18 ml 2, 2, 4- trimethyl pentane containing 1% (v/v) octan-1-ol; Each run lasted 15 minutes with approximately 5 minutes between each run.

are controlled by diffusion of reactants and/or products between the bulk medium and the immobilised enzyme support. Hence, kinetic evaluations made in the presence of external mass transfer limitations give a biased impression of the catalyst's performance.

Essentially, in the situation of mass transfer limitation of a reaction, the transfer of substrate to the catalyst occurs at a rate which is lower than the potential, kinetic rate of conversion of substrate by the catalyst. Hence, kinetic evaluations made under such conditions do not reveal the true kinetic constants of an immobilised enzyme.

External mass transfer limitation of a reaction can be detected by increasing the agitation rate of the reaction medium. In such investigations increase in the agitation rate functions to promote convective transfer of substrate to the immobilised enzyme support thereby reducing diffusional effects until no further increase in reaction rate with stirrer speed is observed. At this point the rate of reaction is controlled either by the rate of diffusion of the substrate/products within the catalytic matrix or is kinetically controlled.

In this case the investigation of mass transfer limitations involved variation of the aqueous and organic phase stirrer speeds. Both the phases were stirred: the organic phase using a magnetic stirrer; and the aqueous phase using an impeller. The effect of stirrer speed was investigated over the range of 0 to 700 R.P.M. for each phase using a fresh, 0.22 μm enzyme-loaded membrane. As the stirrer speed was

varied over several discrete values for one phase the stirrer speed of the other phase was kept constant at approximately 300 R.P.M. Stirrer speeds were measured using a Jacquet DHR 905 tachometer. Each run was carried out in duplicate and the initial run was repeated a third time at the end of the experiment to check the enzyme stability. For each run the change in absorbance at 340 nm of the aqueous phase was monitored for 5 minutes. The aqueous and organic phase compartments of the reactor were washed with buffer and solvent respectively between each run. All reactions were run at room temperature which was measured. Adsorbed protein was recovered per Section 3.3.3.

Figure 3.12 shows the effects of the aqueous and organic phase stirrer speeds on reaction rate. From the graph the aqueous phase stirrer speed was observed to have a profound effect on the reaction rate and the organic phase stirrer speed relatively little effect. This indicated that at low stirrer speeds the reaction was externally mass transfer limited with respect to reaction of NAD^+ or removal of H^+ and/or NADH. Essentially at low stirrer speeds a stagnant layer was present at the membrane/aqueous phase surface and therefore, for example, substrate had to diffuse across this layer to react. Kinetically, the reaction had the potential to proceed faster than the rate of substrate diffusion and so the reaction was diffusion controlled.

In the experiments undertaken as concentration of NAD^+ was essentially unchanged over of the course of the reaction so with respect to NAD^+ the situation could be approximated mathematically as:

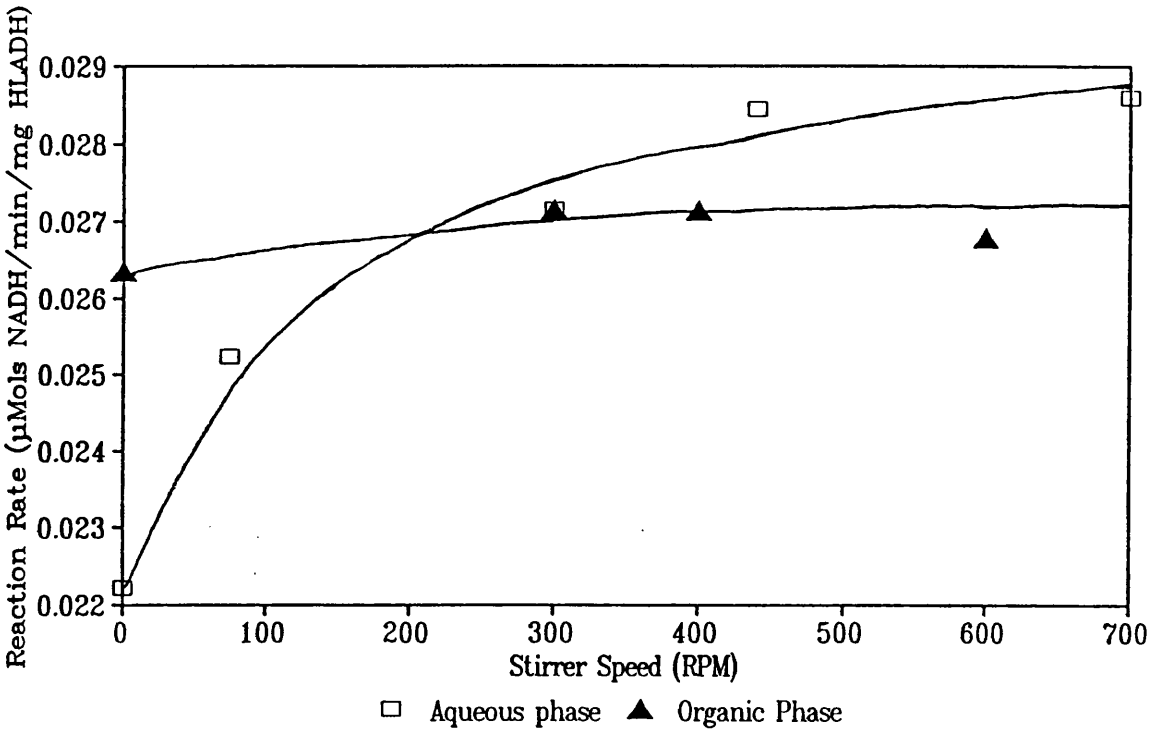


Fig. 3.12. The Effect of the Aqueous and Organic Phase Stirrer Speeds on the Activity of the Immobilised HLADH. (the stirrer speed of one phase was held constant @ 300 RPM whilst the stirrer speed of the other phase was varied)

$$v=\frac{Vmax.S}{Km+S}=K_La(S_0-S)$$

where: S_0= concentration of NAD^+ in the bulk aqueous phase; S= concentration of NAD^+ at the membrane surface; K_La= the mass transfer coefficient; Vmax= maximal velocity of the reaction; Km= Michaelis-Menten constant; v= velocity of the reaction. The equation assumes steady state ie. rate of transfer equals rate of reaction. A similar analysis could be carried out for product escape.

As the aqueous phase stirrer speed was increased the stagnant layer thickness was reduced, S tended to S_0 and so the reaction tended to change from external mass transfer control to kinetic/internal mass transfer control. Kinetic/internal mass transfer control became dominant at approximately 300 R.P.M. as evidenced by the attainment of a stable reaction rate above this stirrer speed. So, with the effects of external mass transfer limitations in mind the aqueous phase stirrer speed was maintained at 350 R.P.M. for all experiments to separate this effect as an influence on results. Although the organic phase stirrer speed appeared to have no significant effect upon the reaction rate stirring of the phase was maintained to prevent the possible formation of concentration gradients.

It must be noted that the above explanation of these results could also be equally true if the reaction was controlled by external mass transfer limitation of product escape from the membrane (eg. if an enzyme was sensitive to product inhibition). In actual fact the relative importance of substrate and product diffusion

to the external mass transfer limitations observed were not discerned.

3.5 SUMMARY

The small glass reactor was designed principally to allow rapid, kinetic analysis of the immobilised enzyme. However, in the early stages of method development the rig was used to examine the effectiveness of the immobilisation process and to determine the potential of the two-phase reaction scheme. Results of initial experiments (Figures 3.2 and 3.3) allowed examination of both of these factors.

The results demonstrated the initial success of retention of enzyme activity upon immobilisation in the two-phase system. Also from this experiment the problem of enzyme desorption was detected and the immobilisation protocol modified (by the addition of a further washing procedure using an NAD^+ solution) to prevent its reoccurrence.

The results of this preliminary investigation also indicated that the reactor could be used to perform initial rate determinations on the immobilised enzyme. This technique was favoured as by its nature it allowed rapid analysis of enzyme activity. Also, using this technique it was possible to carry out a parallel analysis of soluble enzyme activity.

Factors which had the potential to introduce variation into measurements made

in this fashion were investigated eg. enzyme stability and the aqueous and organic phase stirrer speeds. From these experiments suitable experimental conditions were established for use in initial rate determinations of immobilised enzyme.

CHAPTER 4

KINETIC DETERMINATIONS USING SOLUBLE ALCOHOL DEHYDROGENASE IN A SINGLE PHASE, AQUEOUS SYSTEM

4.1 INTRODUCTION

A series of experiments were designed and undertaken to examine the effects of immobilisation and the presence of the two-phase system on the activity of HLADH. The investigation was undertaken in two parts. First, reported in this chapter, the soluble enzyme was systematically characterised in terms of kinetics, stability and response to variation of the chemical and physical environment. Secondly, in Chapter 5, where possible kinetic behaviour of the immobilised enzyme was characterised in a similar fashion using the small glass reactor developed in Chapter 3. This approach allowed changes in kinetic behaviour of the enzyme, induced by immobilisation, to be clearly identified.

4.2 DETERMINATION OF THE MICHAELIS-MENTEN CONSTANTS FOR SOLUBLE HLADH

The initial reaction rate of the enzyme was measured by the production of NADH from NAD^+ via monitoring of the increase in absorbance at 340 nm using a Cecil CE 588 spectrophotometer. The reaction conditions used are outlined below.

1.00 ml .....NAD^+ solution in assay buffer

0.15 ml.....Octan-1-ol solution in assay buffer

0.05 ml.....0.25 mg/ml ADH in assay buffer

1.80 ml.....0.1 M NaOH-glycine assay buffer pH 9.5

3.00 ml Total volume

The octan-1-ol solutions were prepared volumetrically by adding an accurately weighed amount of octan-1-ol to a volumetric flask which was then filled to approximately 80% of its capacity with the assay buffer. The octan-1-ol was then dissolved by sonicating the mixture for several hours until no distinct phases were visible. The solution was then adjusted to the required final volume by addition of buffer. This procedure was adopted as the volume of the solution expanded significantly as the octan-1-ol dissolved. As very small amounts of octan-1-ol were dissolved (typically 100 mg/l) the buffer concentration was considered to be unaffected.

NAD^+ and HLADH solutions were stored on ice prior to use. All reagents

NAD^+ and HLADH solutions were stored on ice prior to use. All reagents were added to the cuvette except the enzyme and the reaction mixture was allowed to equilibrate to the assay temperature of 20°C. The reaction was initiated by addition and thorough mixing in of the enzyme solution. Initial reactions rates were measured at 5 different coenzyme concentrations for each of 5 different octan-1-ol concentrations. The substrate concentrations used were established using a method outlined by Wharton and Eisenthal (1981) whereby several initial experiments were performed at very low and very high substrate concentrations and the results graphed as a direct linear plot. This plot allowed substrate concentrations to be selected that would produce roughly equal divisions of initial rates. Concentrations were; 30, 40, 60, 84, 100 μM NAD^+ and 6, 12, 23, 35, 47, 58 μM octan-1-ol. For each set of assay conditions reactions were run in triplicate.

Results were analyzed by estimation of the slope of the initial rate of reaction. The molar extinction coefficient for NADH was taken to be 6220 $l.mol^{-1}.cm^{-1}$ (Dawson et.al. 1986). Michaelis-Menten constants were obtained from the initial rate data obtained at different concentrations of octan-1-ol and NAD^+ were averaged and then analyzed using the direct linear plot method (Eisenthal and Cornish-Bowden 1974) for a two substrate enzyme reaction proceeding via the Theorell-Chance mechanism i.e. the reaction proceeds via ordered binding of NAD^+ then octan-1-ol, but the enzyme/NAD^+/octan-1-ol complex was considered not to accumulate (Rawn 1983, Dalziel 1970).

Velocity of reaction is given by the generalised equation for a two substrate enzyme reaction at steady state velocity:

$$v=\frac{V.a.b}{Ks^{a}.Km^{b}+Km^{b}.a+Km^{a}.b+a.b} \qquad (1)$$

where: v= the velocity observed; V= maximal velocity of the reaction; Ks^{a}= Equilibrium dissociation constant for the NAD^{+}/HLADH complex; Km^{b}= Michaelis-Menten constant for octan-1-ol; Km^{a}= Michaelis-Menten constant for NAD^{+}; a= concentration of NAD^{+}; b= concentration of octan-1-ol.

If the concentration of b (octan-1-ol) is held constant and the concentration of a (NAD^{+}) is varied then Equation 1 becomes:

$$v=\frac{V^{app}.a}{Km^{app}+a} \qquad (2)$$

Where V^{app} the apparent maximal velocity is given by:

$$V^{app}=\frac{V.b}{Km^{b}+b} \qquad (3)$$

and Km^{app} the apparent Michaelis-Menten constant is given by:

$$Km^{app} = \frac{Ks^{a}.Km^{b} + Km^{a}.b}{Km^{b} + b} \qquad (4)$$

so:

$$\frac{V^{app}}{Km^{app}} = \frac{(V/Km^{a})b}{Ks^{a}.Km^{b}/Km^{a} + b} \qquad (5)$$

Using Equation 2 the following initial, direct linear plots were drawn: v against the concentration of NAD^+ (a) for each concentration of octan-1-ol (b), to give values of V^{app} and Km^{app} for each concentration of octan-1-ol. Figure 4.1 shows an example plot. Next, a subsequent plot of V^{app} against the concentration of octan-1-ol (b) was drawn using the values of V^{app} obtained from the initial direct linear plots. From Equation 3 this plot (Figure 4.2) gave values of V and Km octan-1-ol (Km^b). A final plot (Figure 4.3) of Vm^{app}/Km^{app} against octan-1-ol concentration (b) gave values of V/Km^a and $Ks^a.Km^b/Km^a$. Hence, using Equation 5 the value of Km NAD^+ (Km^a) was obtained. The estimates of the kinetic constants obtained from the direct linear plots are shown in Table 4.1.

Values obtained for the kinetic constants were then used to calculate theoretical values of initial rates of reaction using the equation for two substrate Michaelis-Menten kinetics. Rates were calculated at all concentrations of NAD^+ and octan-1-ol that were used experimentally. The experimental and theoretical results were plotted as graphs of initial reaction rate against substrate (NAD^+) concentration to allow a visual comparison. These plots are shown as Figures 4.4 and 4.5. From these graphs

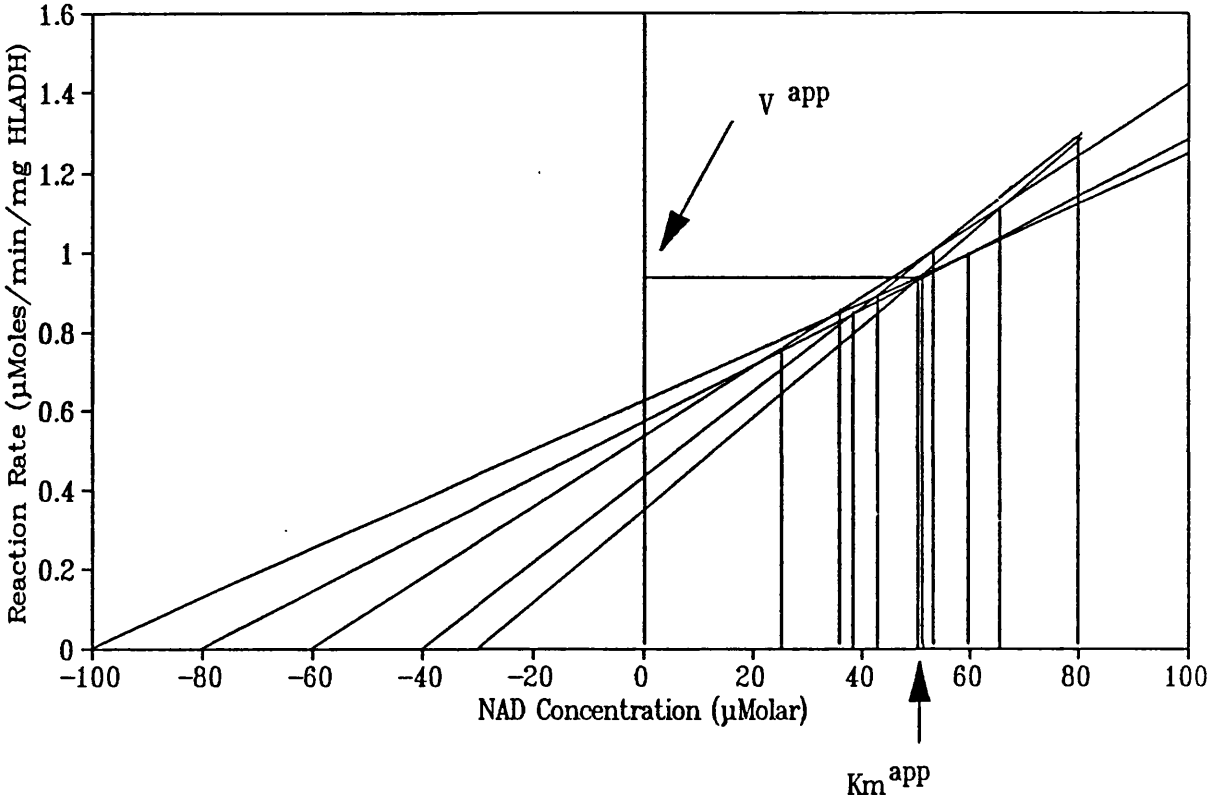


Fig 4.1. An Example Initial Direct Linear Plot to Obtain V^{app} and Km^{app} for soluble HLADH Octan-1-ol= 47 µM

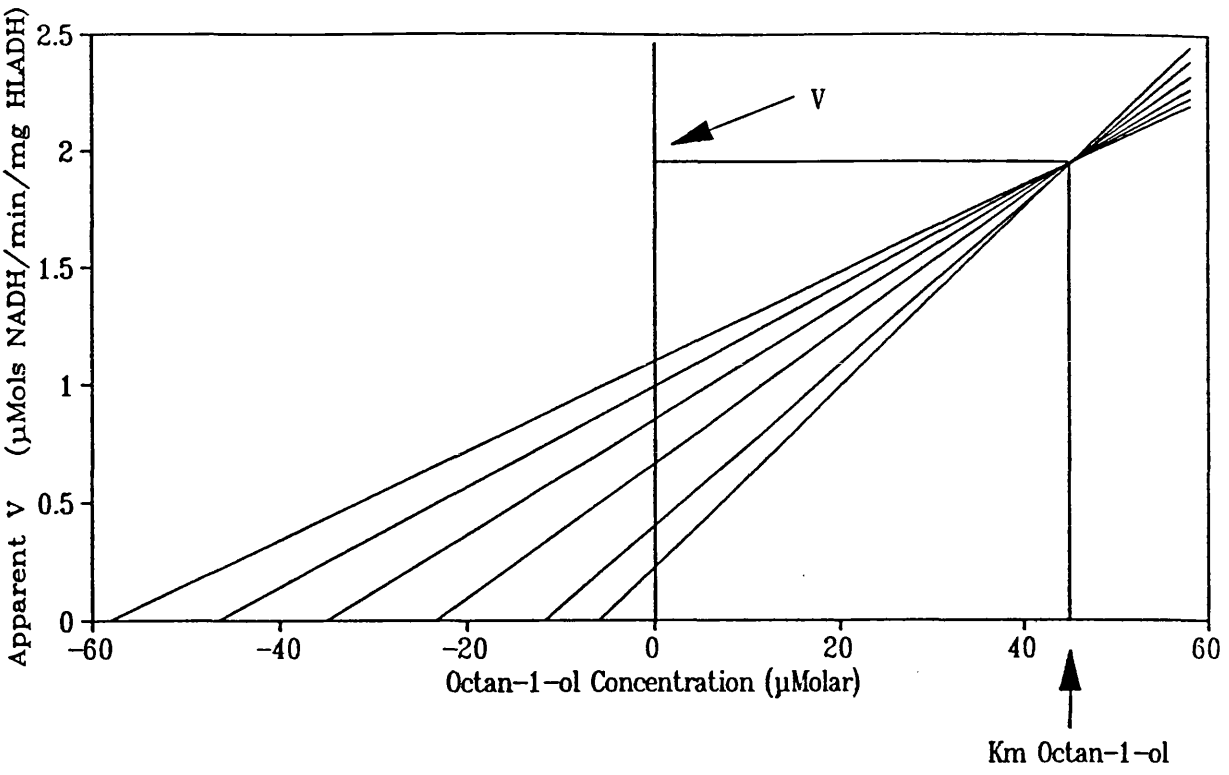


Fig 4.2. A Secondary Direct Linear Plot of V^{app} against Octan-1-ol Concentration to Obtain Km Octan-1-ol and V for soluble HLADH

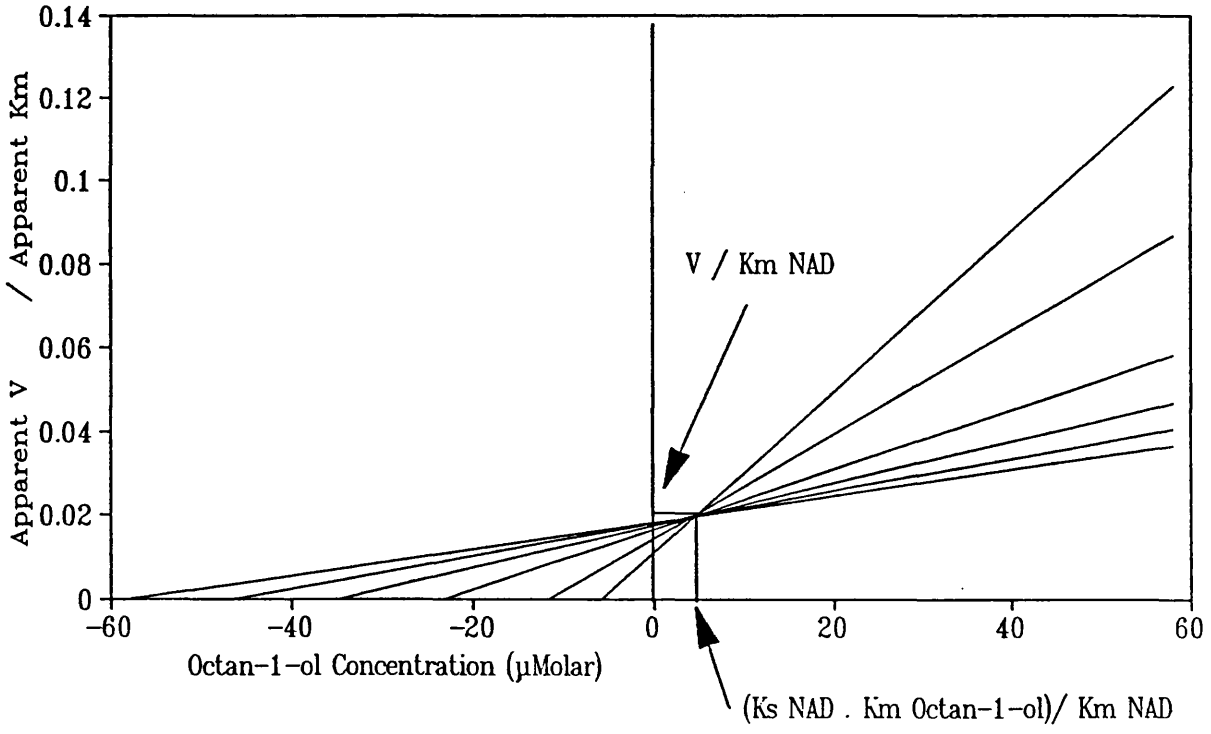
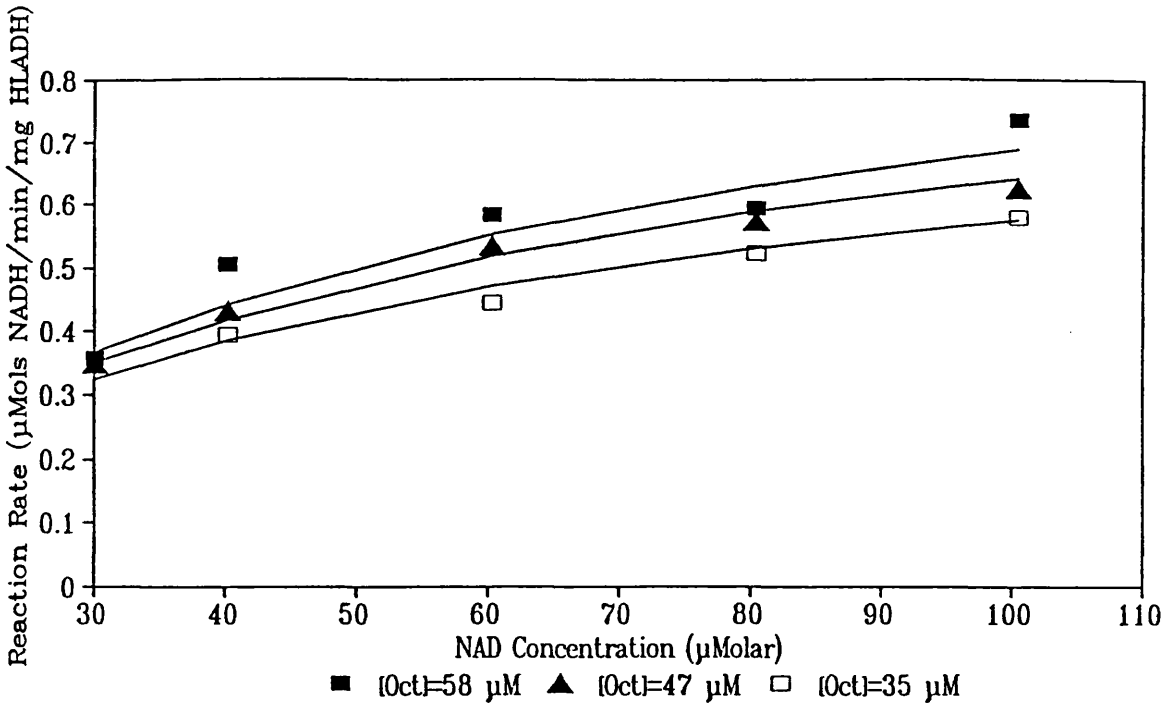
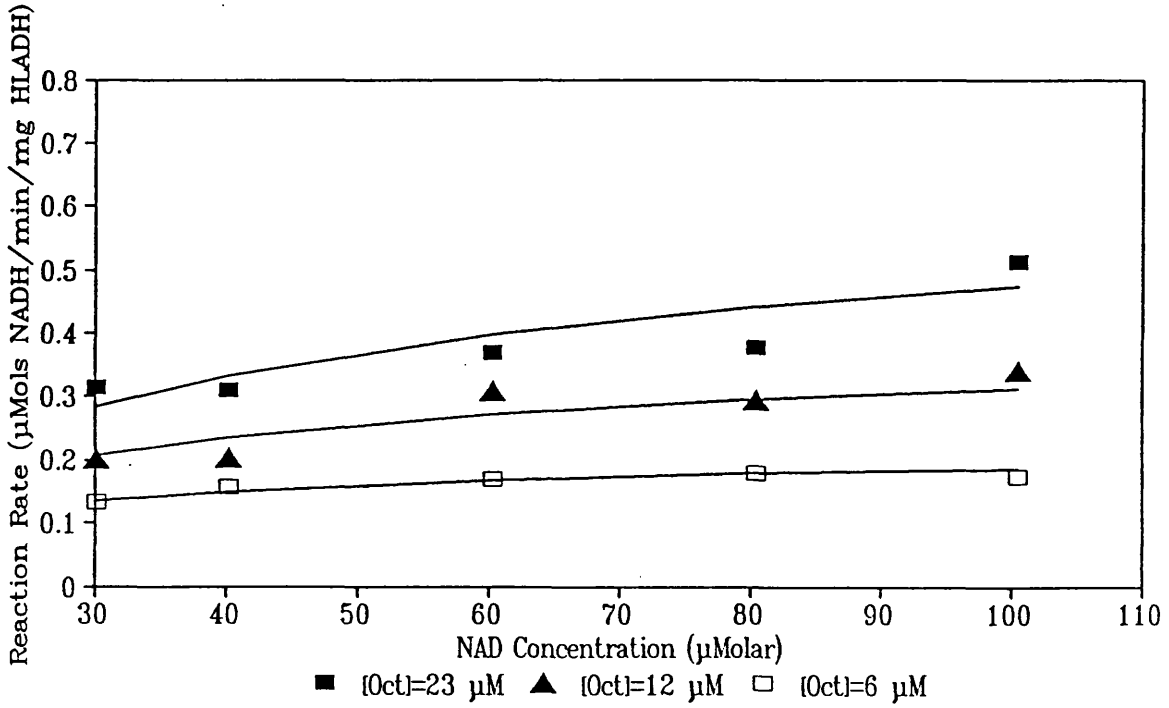


Fig 4.3. A Direct Linear Plot of V^{app} / Km^{app} against Octan-1-ol Concentration to Obtain Km NAD for soluble HLADH.



The solid lines show theoretical curves for the data produced using the DLP estimates of the kinetic constants.

Fig 4.4. Reaction Velocity versus NAD Concentration for Soluble HLADH at Different Concentrations of Octan-1-ol.



The solid lines show theoretical curves for the data produced using the DLP estimates of the kinetic constants.

Fig 4.5. A Continuation of Figure 4.4. Reaction Velocity versus NAD Concentration for Soluble HLADH at Different Octan-1-ol Concentrations.

Kinetic Constant	Value Obtained
Km NAD^+ μMolar	99
Km Octan-1-ol μMolar	45
Ks NAD μMolar	0.85
V μMoles/min/mg HLADH	1.96

Table 4.1 Kinetic Constants of the Free Enzyme at 20°C

it can be seen that theoretical plots closely mirrored experimental ones indicating the Michaelis-Menten model provided an excellent description of experimentally observed enzyme kinetics.

A more critical method of analysis of enzyme kinetic behaviour was provided by a linear plot of the Michaelis-Menten equation. Several forms were available, the linear form chosen in this case was an Eadie-Hofstee plot of initial reaction rate divided by the substrate concentration (NAD^+) plotted against the initial reaction velocity ie. v/S versus v. This type of linear plot is not often used in enzyme kinetic studies because even data containing only small, random experimental errors can produce plots which deviate considerably from linearity. Figure 4.6 highlights this and shows the relatively wide error bars associated with this type of plot.

In this case, it was intended to compare results for the soluble HLADH to those obtained for HLADH immobilised in a two-phase system. As immobilised enzymes can show marked systematic deviations from Michaelis-Menten kinetics induced by such mechanisms as mass transfer limitations and partition effects (Bailey and Olis 1977) the use of Eadie-Hofstee plots provides a sensitive test for systematic deviations from Michaelis-Menten kinetics. Hence, by comparison of these plots for the soluble and immobilised enzyme any deviation from Michaelis-Menten kinetics by the immobilised enzyme could be observed and identified as a direct consequence of immobilisation.

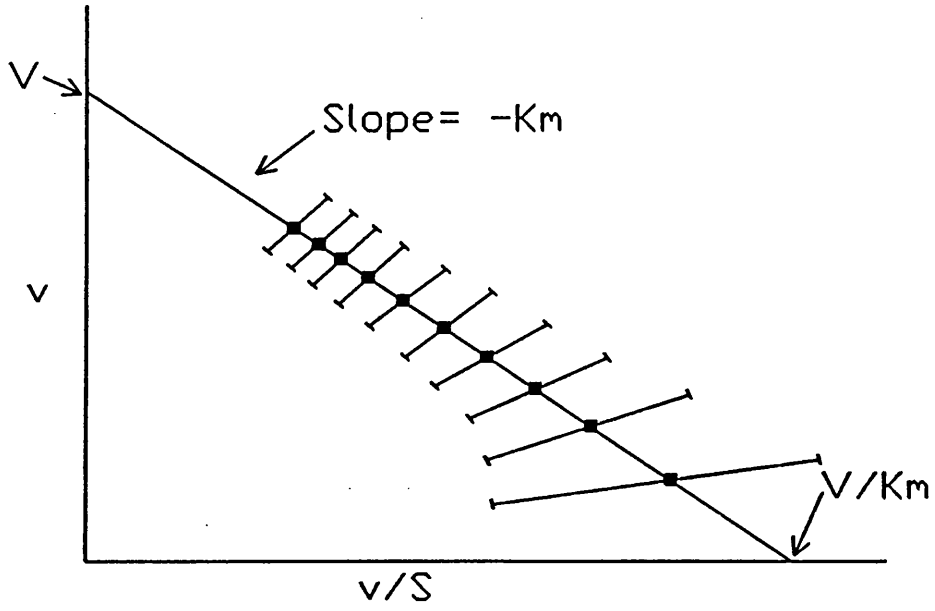


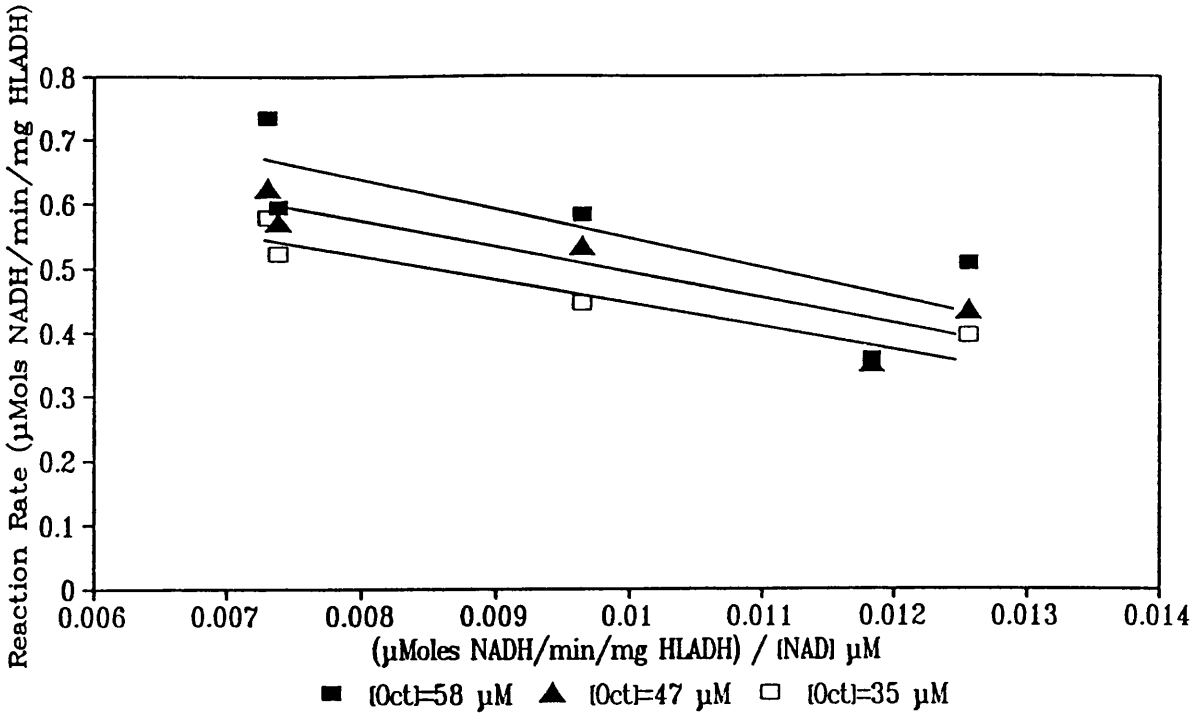
Fig 4.6 Eadie-Hofstee Plot with Error Bars of 0.05 V in v to Show How Small Errors in Experimental Results can Lead to Large Devations from Linearity
From Cornish-Bowden (1979)

Eadie-Hofstee plots for the soluble enzyme are shown as Figures 4.7 and 4.8. From these plots it can be seen that no systematic deviations from Michaelis-Menten kinetics were apparent. In fact, relatively small, random errors were the only source of deviation from the Michaelis-Menten results. It was concluded that the soluble enzyme had demonstrated an accurate fit to Michaelis-Menten kinetics and the experimental estimates of the kinetic constants were accurate.

4.3 DETERMINATIONS OF THE EFFECT OF pH ON ACTIVITY OF SOLUBLE HLADH

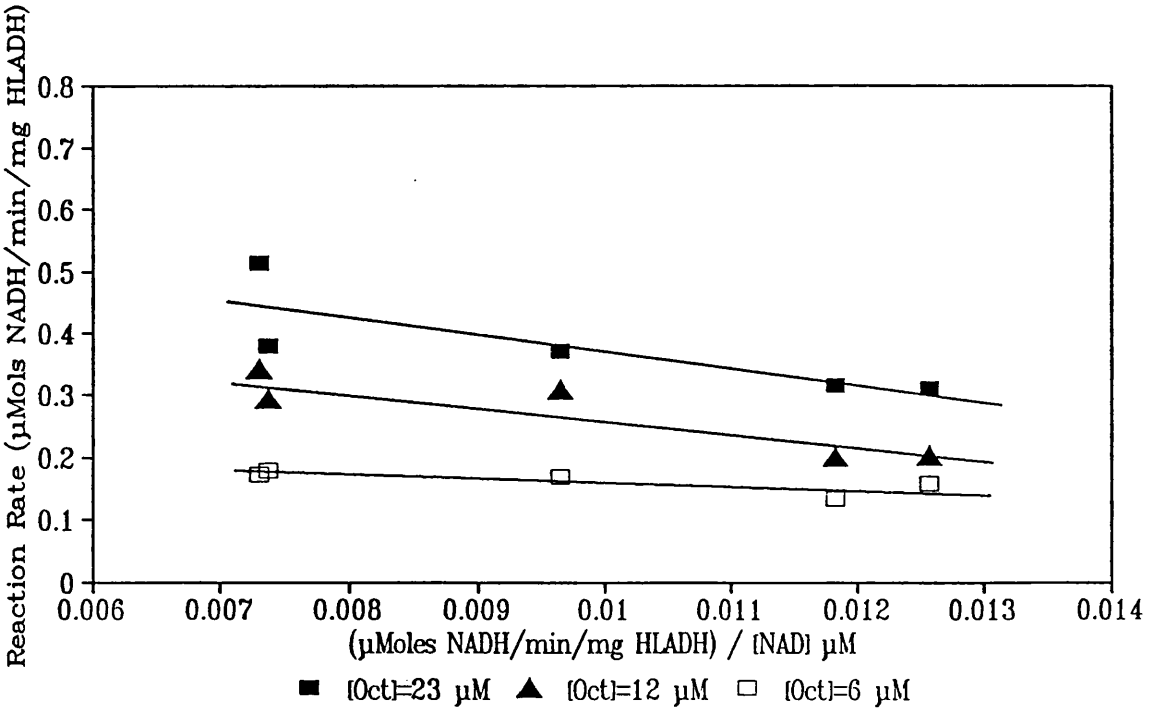
Again initial rates of reaction were measured. Reaction conditions and protocols were the same as in Section 4.2 above except: 1) for all experiments the NAD^+ solution used was 1 mg/ml and the octan-1-ol solution used was 104.5 mg/l and; 2) the pH of the assay buffer was varied at pH 8.5, 9.0, 9.5, 10.0, and 10.5. Samples were run in triplicate at each pH. The assay conditions were based on those of Dalziel (1957) except octan-1-ol was the substrate rather than ethanol. Additionally, the pH range was formulated taking account of the fact that: 1) as the pH is lowered towards neutrality the reaction equilibrium favours the formation of the alcohol (Brändén et.al. 1975) and; 2) at very high pH (11 or greater) the enzyme was inactivated rapidly.

Saturation of each substrate was checked in each buffer by halving the concentration of both NAD^+ and octan-1-ol in turn. In each case no appreciable



The solid lines show theoretical curves for the data produced using the DLP estimates of the kinetic constants.

Fig 4.7. A Plot of v against v/S (NAD) for Soluble HLADH at Several Concentrations of Octan-1-ol.



The solid lines show theoretical curves for the data produced using the DLP estimates of the kinetic constants.

Fig 4.8. A Continuation of Fig 4.7. A Plot of v against v/S (NAD) for Soluble HLADH at Several Concentrations of Octan-1-ol.

change in initial rate was observed indicating that saturating conditions were achieved.

The pH activity profile of the enzyme is presented as Figure 4.9. The results the showed less than 20% variation in the pH range 8.5-10.5 with the highest rate recorded at pH 9.5.

4.4 DETERMINATION OF THE EFFECT OF IONIC STRENGTH ON THE ACTIVITY OF SOLUBLE HLADH

Reaction conditions and protocols were the same as those described in Section 4.3 except that for each set of triplicate experiments the ionic strength of the assay buffer was varied at 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 M and the pH kept constant at pH 9.5. ie. reaction conditions were:

1.00 ml ...1 mg/ml NAD^+ solution in assay buffer

0.15 ml....104.5 mg/ml Octan-1-ol solution in assay buffer

0.05 ml....0.25 mg/ml ADH in assay buffer

1.80 ml....NaOH-glycine assay buffer pH 9.5

3.00 ml Total volume

The results obtained are displayed as Figure 4.10. The graph profile showed an approximate 5% decrease in HLADH activity with increasing buffer strength. This relationship was approximately linear over the range of ionic strengths used.

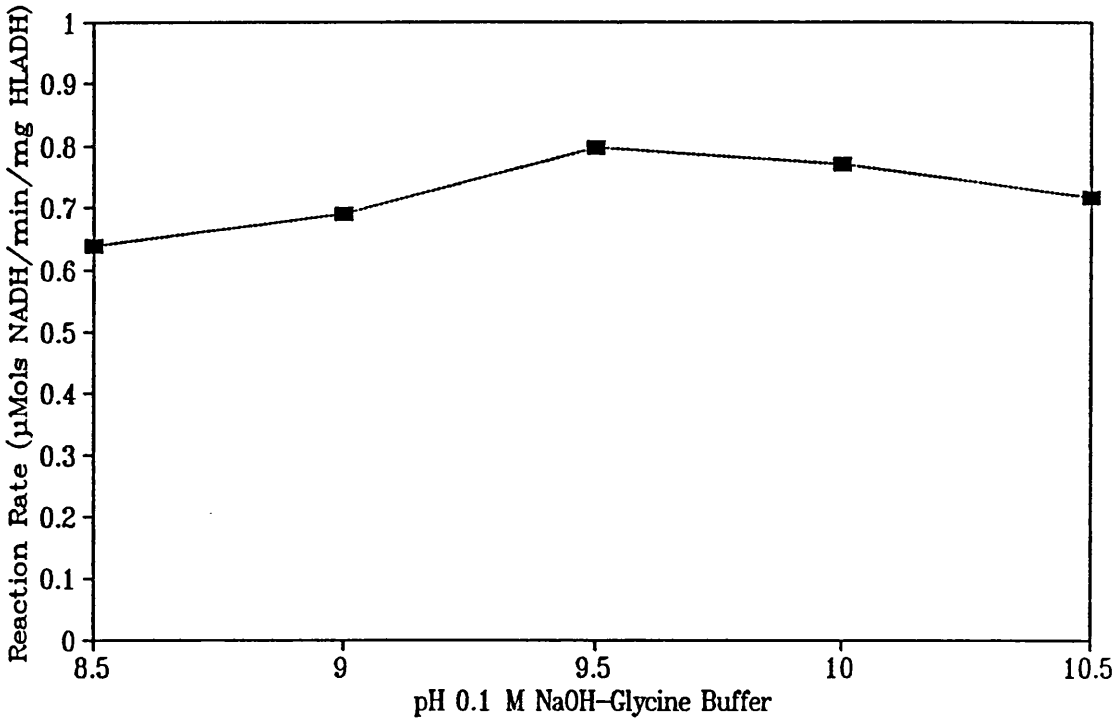


Fig 4.9. The Effect of pH on the Activity of HLADH in Free Solution.

(Temperature= 20°C)

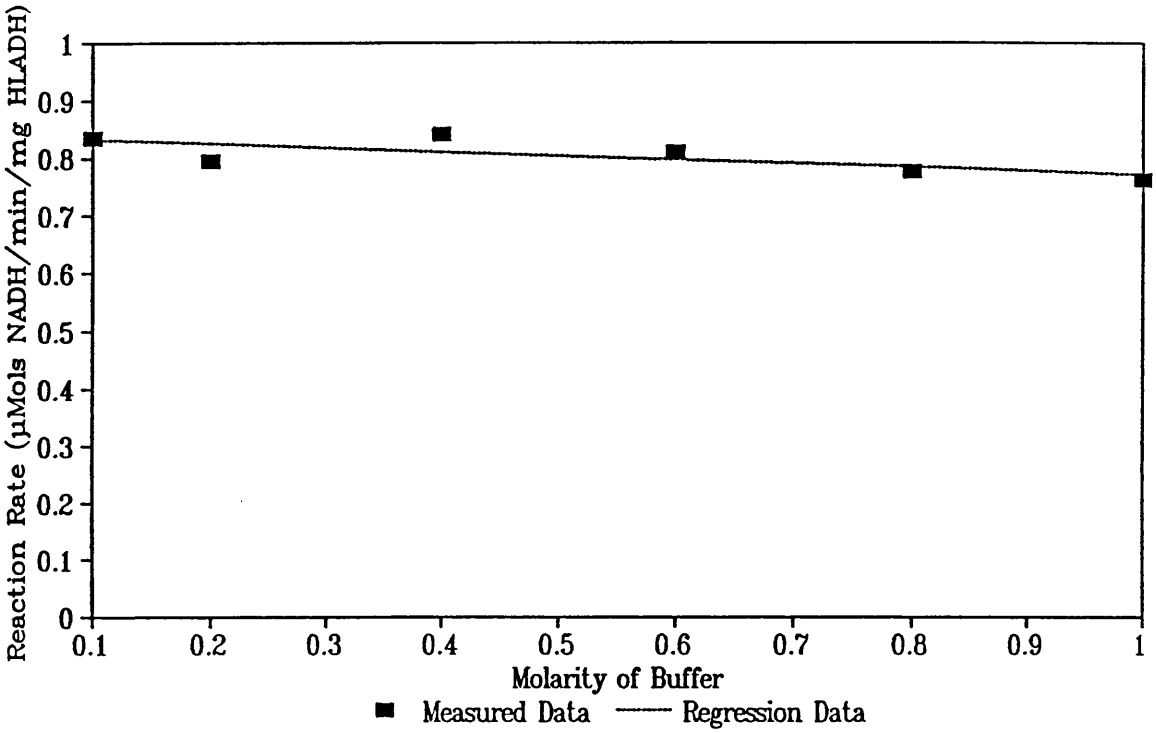


Fig 4.10. The Effect of Ionic Strength on Activity of the Free Enzyme (Temperature= 19°C)

4.5 DETERMINATIONS OF SOLUBLE HLADH STABILITY

1 mg/ml solutions of HLADH were prepared in pH 8.0, 8.5, 9.0, 9.5, 10.0, 10.5 and pH 11.0, 0.1 M NaOH-glycine buffer and triplicate initial reaction rate determinations were made daily for 4 days. The solutions were stored in the dark at room temperature. Reaction conditions were:

1.80-1.70 ml....NaOH-glycine assay buffer pH 9.5

1.00 ml .....1 mg/ml NAD^+ solution in assay buffer

0.15 ml.....104.5 mg/ml Octan-1-ol solution in assay buffer

0.05-0.15 ml....1 mg/ml HLADH in assay buffer

3.00 ml Total volume

The volume of enzyme solution added was varied to produce an accurately measurable initial rate of reaction. The volume of buffer added was adjusted accordingly to maintain the overall volume at 3.00 ml.

Figure 4.11 shows stability profiles of the soluble enzyme at different pH values. The enzyme showed significant loss of stability at all pH values. After 4 days the highest stability was retained at pH 9.5 (approximately 60% of its original activity). Over the range investigated the apparent sequence of stability retention at different pH values, from the highest to the lowest, was pH 9.5 > 8.0 > 11.0.

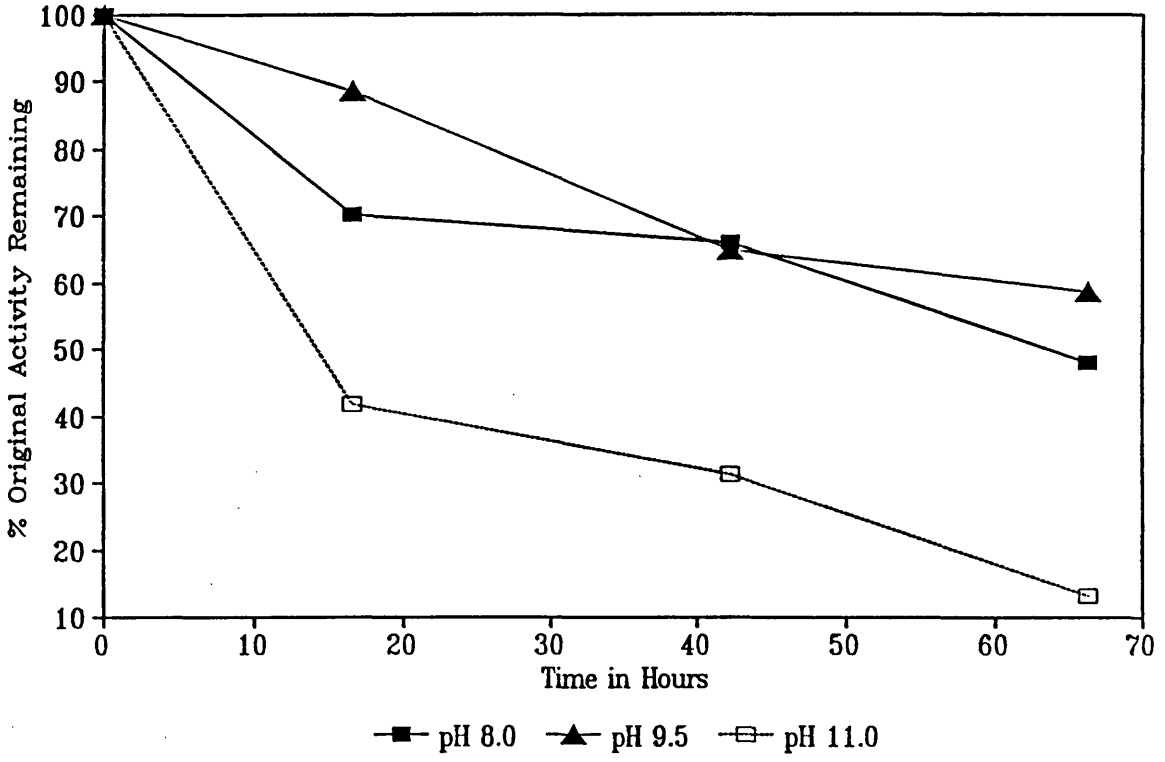


Fig 4.11. The Effect of pH on the Stability of HLADH in Free Solution. (enzyme solutions were stored in the dark @ room temperature- 18-20°C)

4.6 SUMMARY

A series of control results were obtained for comparison to those for the enzyme immobilised in a two-phase system. Specifically, kinetics of the soluble enzyme were found to closely conform to simple, two-substrate Michaelis-Menten kinetics. The enzyme was found to have a maximal reaction rate at pH 9.5 and showed only a slight change in reaction rate with ionic strength. Maximal stability of the enzyme was observed at pH 9.5.

CHAPTER 5

KINETIC DETERMINATIONS UTILISING MEMBRANE BOUND ALCOHOL DEHYDROGENASE IN AN AQUEOUS-ORGANIC TWO-PHASE SYSTEM

5.1 INTRODUCTION

As outlined at the start of Chapter 4 the experiments conducted in this Chapter were conducted to allow comparison to data obtained using soluble HLADH. To this end similar experimental methods were used as far as possible. This allowed a detailed comparison of the soluble and immobilised enzyme systems and evaluation of any changes in activity resulting from immobilisation of the enzyme in a two-phase system.

5.2 DETERMINATION OF THE APPARENT MICHAELIS-MENTEN CONSTANTS FOR IMMOBILISED HLADH

Taking a single, fresh, 0.22 μm enzyme-loaded membrane a series of reactions were run using varied coenzyme concentrations against a fixed octan-1-ol concentration. Concentrations were: NAD^+; 40, 150, 500, 1000, 1500 μM and octan-1-ol; 63, 380, 630, 63000, 632000 μM. The increase in absorbance at 340 nm was

monitored for 5 minutes using the chart recorder program and the data stored on floppy disk for analysis. Each concentration was run in duplicate. Between each run the aqueous and organic phase compartments of the reactor were thoroughly rinsed with 0.1 M, pH 9.5 NaOH-glycine buffer and 2,2,4-trimethyl pentane, respectively. The highest concentration of reactants was run first and then the other runs were carried out in random order. At the end of each series of runs the initial run was repeated a third time to check the enzyme stability over the course of the experiments. The aqueous and organic phase stirrer speeds were set at 350 and 330 R.P.M., respectively.

Following running of a full set of NAD^+ concentrations against a fixed octan-1-ol concentration the membrane was removed and blotted dry. Adsorbed protein was then recovered as described in Section 3.3.3. Using the same membrane (following the membrane regeneration, re-equilibration and immobilisation processes outlined in Sections 3.3.1 and 3.3.2) the whole procedure was repeated at a different fixed octan-1-ol concentration. All reactions were run at room temperature (17-19°C).

Direct linear plots were prepared for analysis of the experimental results in terms of Michaelis-Menten kinetics. The technique is described fully in Section 4.2. Using the Michaelis-Menten constants obtained from this analysis and the two substrate Michaelis-Menten equation, theoretical initial reaction rates were calculated at each concentration of NAD^+ and octan-1-ol used experimentally. Plots of initial reaction rate against substrate concentration (NAD^+) were drawn at each octan-1-ol concentration used for both the experimental and the theoretical results. These plots

are shown as Figures 5.1 and 5.2.

It can be seen from these plots that the experimental results were internally consistent. i.e. at each concentration of octan-1-ol used the results showed an increase in initial reaction rate with increase in NAD^+ concentration and this trend reduced as enzyme saturation was approached. However, when the experimental and theoretical plots were compared then large deviations between these results were apparent at both high (632000 μM) and low (63 μM) octan-1-ol concentrations. At 380 μM, 630 μM and 63000 μM the experimental results agreed closely with the theoretical results. The inference of these observations was that the two substrate, Michaelis-Menten equation provided too limited a framework to describe the experimental results adequately.

Further confirmation of deviation from Michaelis-Menten kinetics was observed using Eadie-Hofstee plots: plots of initial reaction rate divided by the substrate concentration (NAD^+) against the initial reaction rate i.e. plots of v/S against v. As outlined in Section 4.2, this linear form of the Michaelis-Menten equation was used as it is the most sensitive of this type of plot to detection of systematic deviations between results and the underlying model. In this case, it was reasoned that if the kinetic behaviour of the immobilised enzyme was inadequately described by the Michaelis-Menten equation then experimental results plotted as v/S against v would display systematic (rather than random) deviations from linearity. Eadie-Hofstee plots for the immobilised HLADH are shown as Figures 5.3 and 5.4.

From these graphs it can be seen that the plots displayed a curvature at each

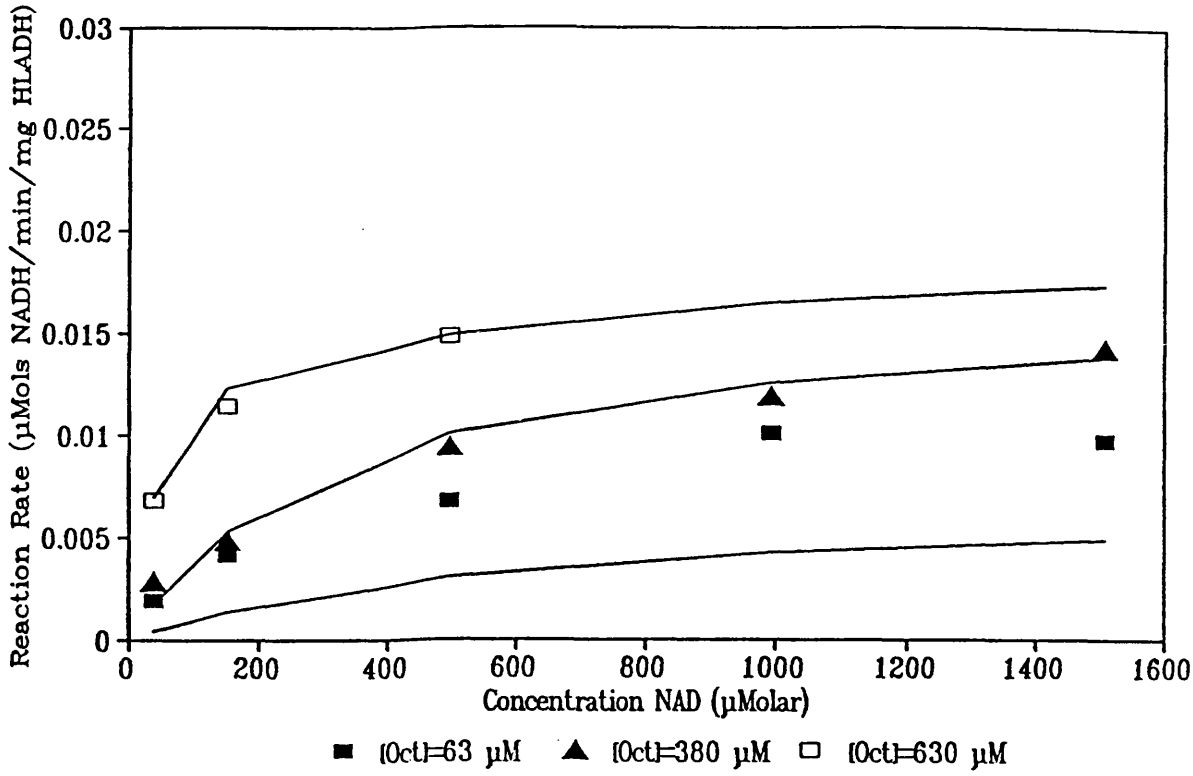


Fig 5.1. Reaction Velocity versus NAD Concentration for Immobilised HLADH at Different Concentrations of Octan-1-ol.

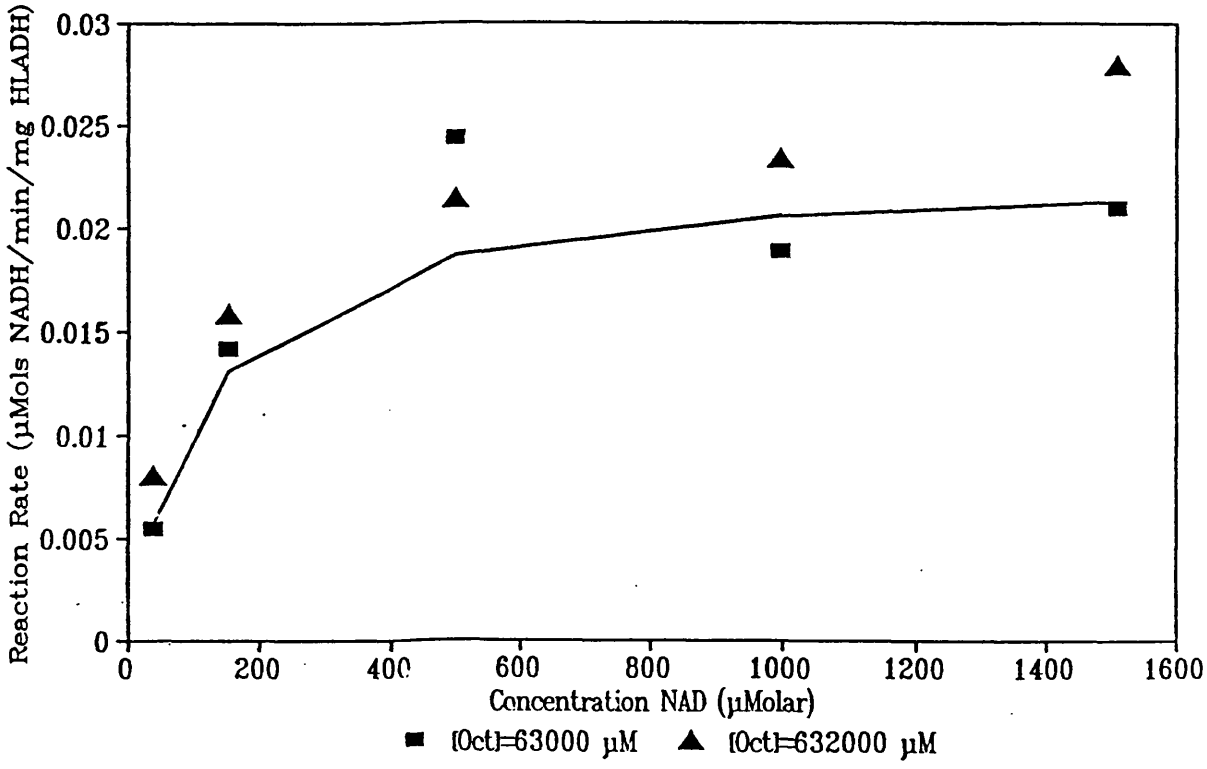


Fig 5.2. A Continuation of Fig 5.4. Reaction Rate versus NAD Concentration for Immobilised HLADH at Different Concentrations of Octan-1-ol.

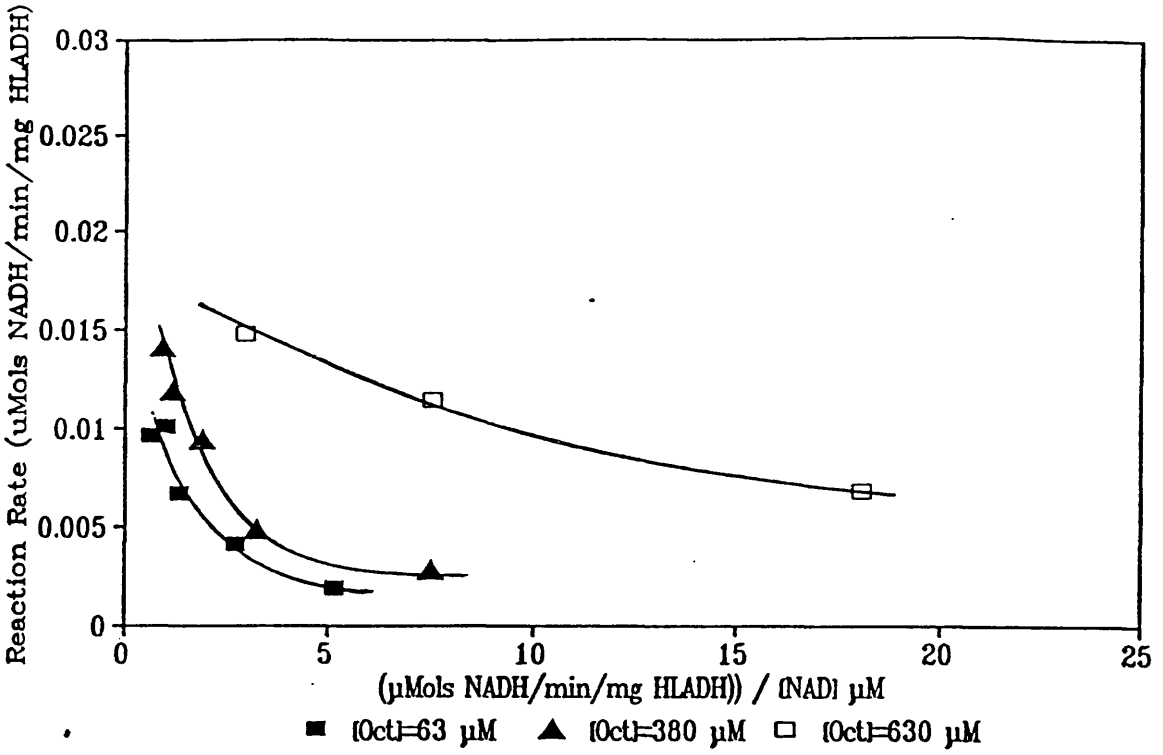


Fig 5.3. A Plot of v against v/S (NAD) for Immobilised HLADH at Several Concentrations of Octan-1-ol

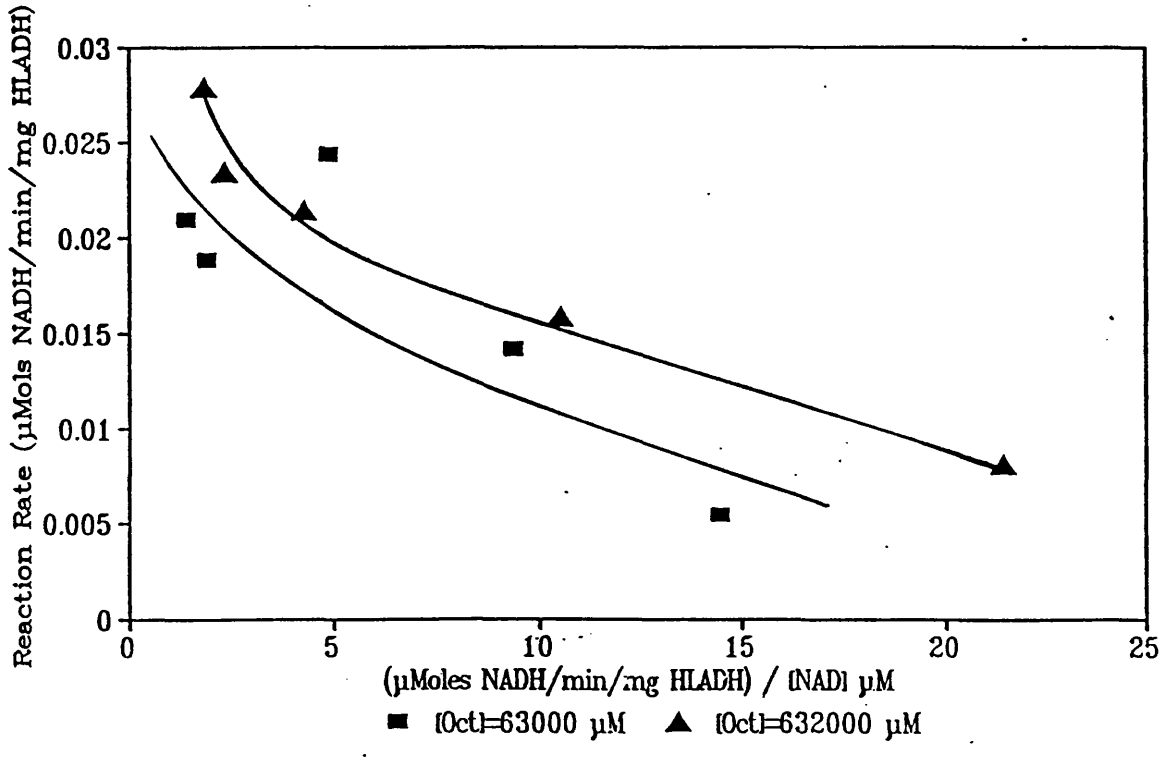


Fig 5.4. A Continuation of Fig 5.6. A Plot of v against v/S (NAD) for Immobilised HLADH at Several Concentrations of Octan-1-ol

octan-1-ol concentration. This is most clearly observed at in the plots at 63 μM and 380 μm octan-1-ol As the octan-1-ol concentration was increased then the effect reduced in intensity and is less obvious at higher octan-1-ol concentrations. This suggested that deviation from Michaelis-Menten kinetics was to some degree concentration dependant and that concentration gradients were established between the membrane and the bulk phases. Clearly, these plots demonstrated non Michaelis-Menten behaviour of the immobilised enzyme and the inadequacy of the two substrate Michaelis-Menten equation to fully describe the experimental results.

For completeness only, the Michaelis-Menten constants derived for the immobilised enzyme are included as Appendix 2.

Variation from the Michaelis-Menten kinetic model has often been observed for immobilised enzyme systems (Bailey and Ollis 1977). Physical or chemical containment of an enzyme at a solid surface has the potential to modify many aspects of an enzyme's kinetic behaviour. The mechanisms responsible include: charge interaction of the substrate/product/enzyme with the support; partitioning of product or substrate between the support and the bulk solution; steric hinderance of substrate binding and product escape and mass transfer limitations. From the information obtained in this experiment alone it was not possible to determine which of these mechanisms was producing the deviation from Michaelis-Menten kinetics, although external mass transfer limitation was ruled out by the use of non rate limiting stirrer speeds (see Section 3.4.6).

Factors controlling immobilised enzyme kinetics can be identified by examination of the kinetic response of the enzyme to systematic physical and chemical changes. To this end, investigations were carried out to identify the effects of pH and ionic strength on immobilised HLADH. In a further experiment stability of the immobilised enzyme was established. These experiments allowed comparison of the soluble and immobilised enzyme to be made and in this way, reasons for deviation of the immobilised enzyme system from Michaelis-Menten kinetics were more clearly defined.

5.3 EFFECT OF pH ON INITIAL REACTION RATES OBTAINED USING IMMOBILISED HLADH

Fresh, 0.22 μm, enzyme-loaded membranes were used. The membranes were subject to reaction with 18 ml, 1% octan-1-ol (v/v) in 2,2,4-trimethyl pentane as an organic phase and 1 mg/ml NAD^+ in 15 ml 0.1 M, NaOH-glycine buffer as an aqueous phase. The pH of the buffers used for the aqueous phases were; 8.5, 9.0, 9.5, 10.0, 10.5, 11.0. The increase in absorbance at 340 nm was monitored for 5 minutes. All reactions were run in duplicate at room temperature which was measured. The pH 9.5 buffer was used first, then the other buffers were used in random order. The aqueous and organic phase compartments of the reactor were washed between each run with buffer and solvent respectively. The aqueous and organic phase stirrer speeds were set at 350 and 330 R.P.M. respectively. At the end of the series of runs the pH 9.5 buffer solution was used a third time to validate the

enzyme stability over the course of the experiments. Adsorbed protein was recovered as described in Section 3.3.3.

The results obtained are plotted in Figure 5.5. The graph shows that reaction rate increased in an approximately linear fashion with pH over the range used and no optimum pH was observed. In other duplicate experiments the range was extended to pH 11.5. In these experiments a continuation of the increase of reaction rate with pH was observed. However, results of this type could only be produced for the first experiment at pH 11.5 after this initial experiment the rates of reaction obtained at this pH were significantly lowered suggesting the high pH had resulted in the loss of the enzyme's catalytic activity.

Figure 5.6 shows a comparison of the results for the soluble and immobilised enzyme. Although the initial reaction rates of the two systems varied widely it was considered valuable to examine the trends of the data. The contrast of the results was quite marked; whilst the soluble enzyme showed a maximum reaction rate at pH 9.5, the immobilised enzyme produced a continued increase in reaction rate with pH.

The organic phase substrate and product (octan-1-ol and octyl aldehyde respectively) were non polar compounds and essentially uncharged. Therefore, charge modification (ie. change in pH) was unlikely to have had a substantial effect on these components. Hence, the response to pH was probably produced by an interaction of the membrane environment with the aqueous phase substrate and products (NAD^+, H^+ and NADH respectively).

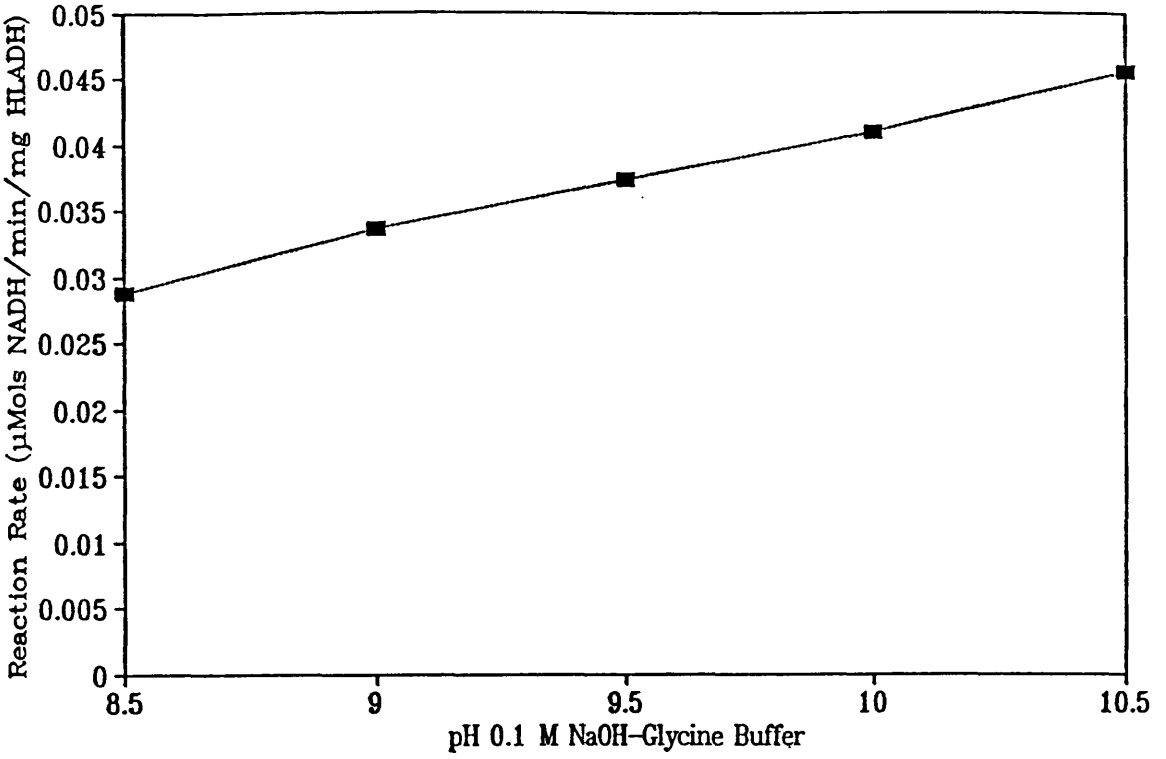


Fig 5.5. The Effect of pH on the Activity of Immobilised HLADH.
(Temperature= 18°C)

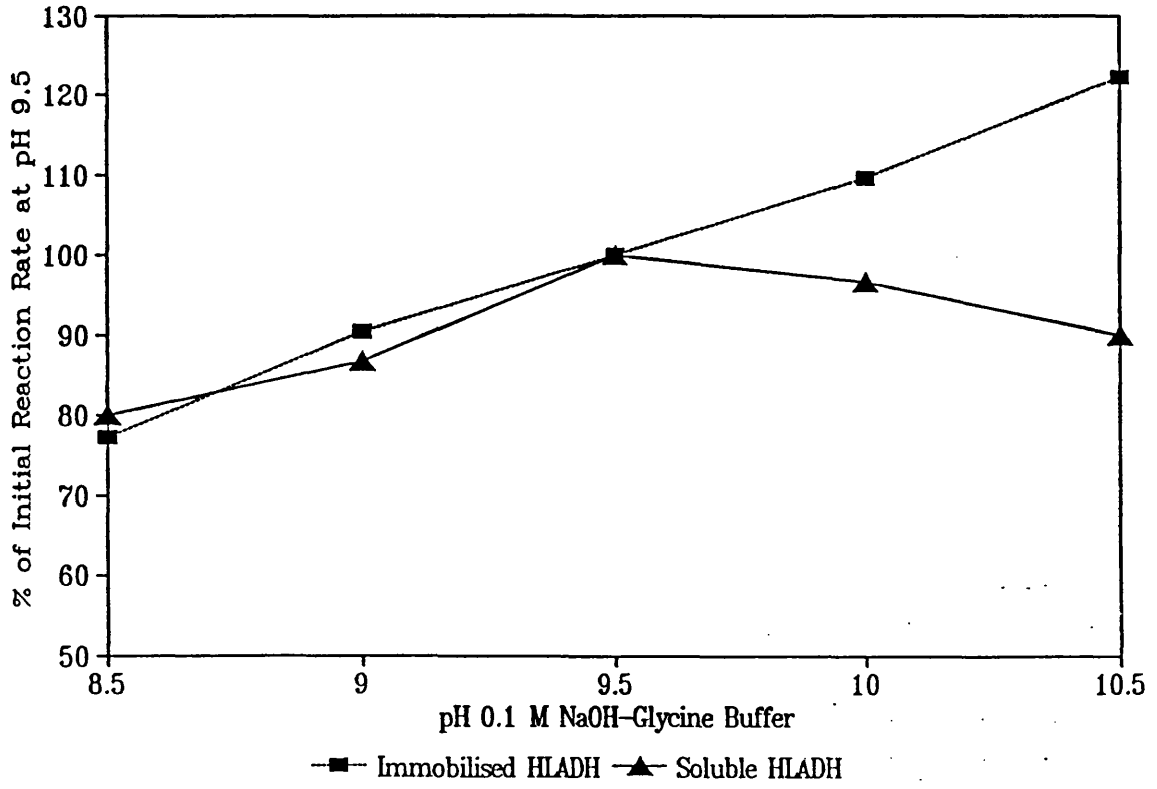


Fig 5.6. The Relative Effects of pH on the Activity of Both Soluble and Immobilised HLADH. Expressed as a percentage of the initial reaction rate at pH 9.5.

The net effect of this interaction was that the internal pH of the membrane (and so the local pH of the enzyme) was maintained at a value lower than that of the bulk aqueous phase. Increasing the pH of the bulk aqueous phase increased the internal membrane pH and therefore the observed reaction rate. Further, comparison of pH plots for the soluble and immobilised enzyme suggested that the internal membrane pH did not exceed pH 9.5 as no pH optimum was observed for the immobilised enzyme.

In essence the pH profile of the immobilised enzyme was "shifted" to a more acidic value relative to the pH profile of the soluble enzyme. As a pH maximum for the immobilised enzyme was not observed the magnitude of this shift was probably at least one pH unit.

There were two possible mechanisms which could have created this effect. The first was microenvironmental effects, this includes charge interactions between the surface of the membrane, the enzyme, the aqueous phase substrate and the aqueous phase products. Presence of a net charge on the membrane would have caused the selective accumulation or exclusion of charged species (NAD^+(-1), NADH(-2) and H^+ions(+1)) within the membrane and so could have altered the intramembrane pH. For example, a net negative charge on the membrane could have lead to an increase in the concentration of H^+ ions within the membrane and so lowered the intramembrane pH.

It is important to note that although the oxidised form of nicotinamide adenine

dinucleotide is given the conventional notation NAD^+, both phosphate groups have pK values below 3 and will be completely ionised conferring a charge of -2 to the molecule, the nitrogen of the nicotinamide ring will also be charged (as an ammonium group) conferring a charge of +1, so the overall charge of "NAD^+" will be -1. Further, when in the reduced form (NADH) the nicotinamide ring losses its charge and so NADH has a net charge of -2.

The second mechanism was internal mass transfer limitation where access of the charged substrates and products within the membrane was limited to diffusion processes. Hence concentration gradients could have developed and again altered the intramembrane pH. The relative importance of these mechanisms to control of the reaction rate of the immobilised enzyme will be assessed in the discussion (Chapter 8).

5.4 EFFECT OF IONIC STRENGTH ON INITIAL REACTION RATES OBTAINED USING IMMOBILISED HLADH

Conditions were the same as those described above for the effect of pH on the initial reaction rate except the pH was held constant at pH 9.5 and the ionic strength of the aqueous phase varied. Concentrations of buffer used were 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 M. The buffer was used for the immobilisation and membrane washing procedures to avoid the possibility of enzyme desorbing from the membrane during the experiments. The experiment using 0.1 M buffer was run a third time at the end

of the experiments as a check the enzyme stability.

Figure 5.7 shows that increasing ionic strength reduced enzyme activity linearly in the range 0.1 to 0.6 M. Over this range the enzyme was reduced in activity by a factor of approximately 25%. Beyond 0.6 M and up to 1.0 M increasing ionic strength had little effect on reaction rate.

The comparative effect of ionic strength is shown in Figure 5.8 where it can be seen that both the immobilised and soluble enzyme showed a reduction in activity with increasing ionic strength. However, the immobilised enzyme showed a greater proportional decrease in activity than the soluble preparation.

Relatively high ionic strength buffers have the property of suppressing charge interactions by charge nullification. In the case of the immobilised enzyme increasing the ionic strength of the buffer would have lead to partial or complete neutralisation of charged species on the membrane or in solution. Hence increasing the ionic strength had the property of modifying the intramembrane pH by the nullification of charged groups created by microenvironmental effects or internal mass transfer limitations.

Finally, comparison of the results for the investigation of pH and ionic strength produced an interesting contrast. The experiment on the effect of pH suggested the intramembrane pH to be "shifted" to a more acidic value relative to the pH 9.5 bulk aqueous phase buffer. If this was so then an increase in the ionic strength

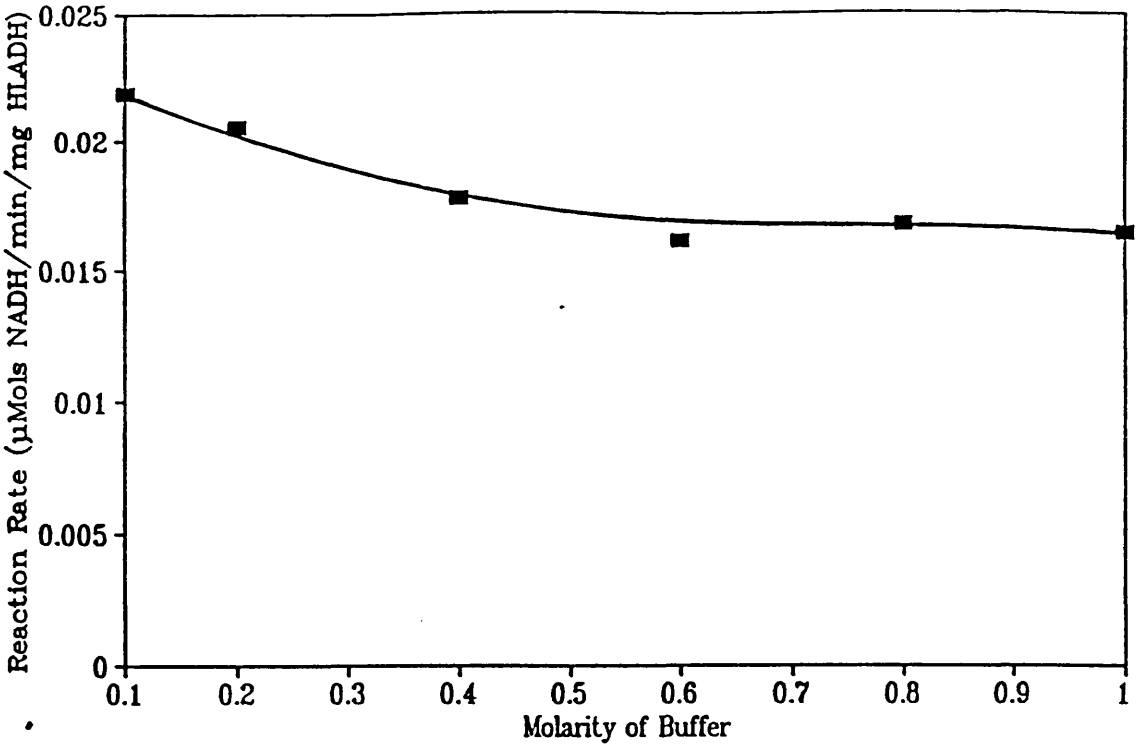


Fig 5.7. The Effect of Ionic Strength on Activity of the Immobilised Enzyme

(Temperature= 18°C)

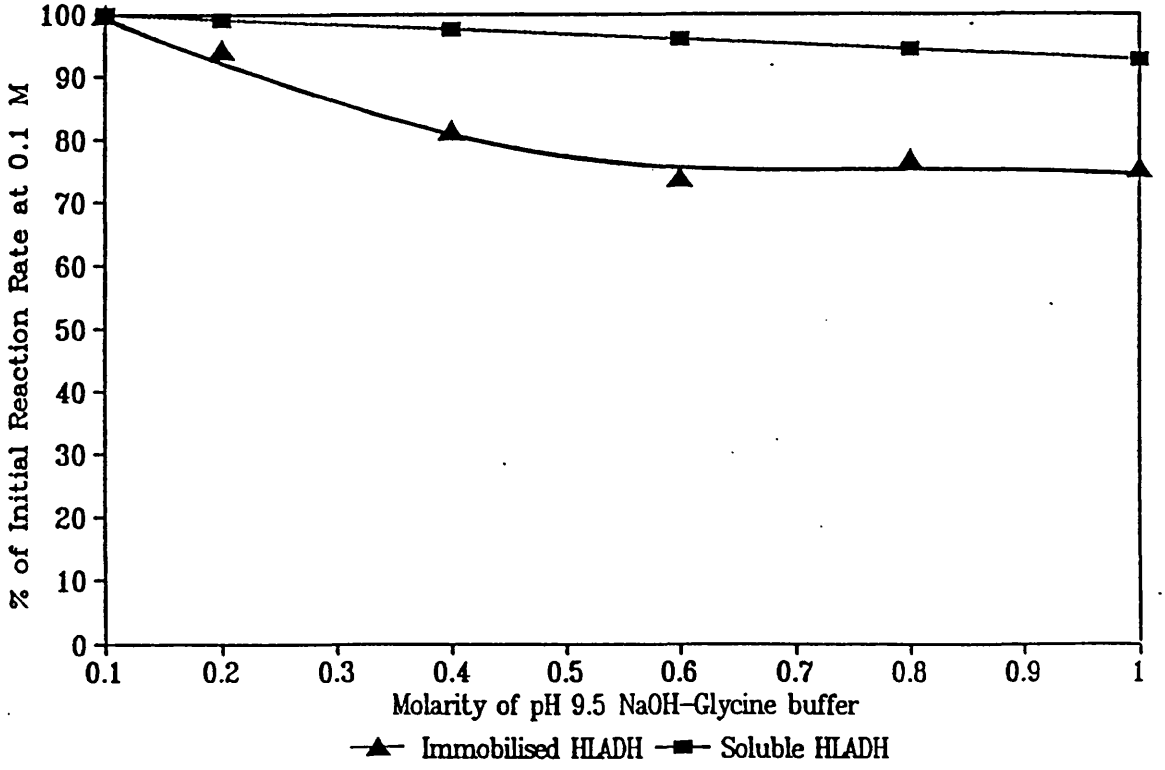


Fig 5.8. The Relative Effects of Ionic Strength on the Activity of Both Soluble and Immobilised HLADH Expressed as a percentage of the inital raction rate at 0.1 M

at pH 9.5 (as was done in this experiment) might have been expected to have nullified this pH shift, returned the intramembrane pH to approximately 9.5 and thereby increasing the observed reaction rate (as expected from the results for the effect of pH). However, this did not occur and increasing the ionic strength at pH 9.5 actually decreased the observed reaction rate. This indicated that the interaction of the substrates and products with the membrane environment was not a simple one and that the pH and ionic strength effects observed were net effects. This can be easily appreciated when it is understood that all the aqueous phase substrate and products were all charged and all of these compounds had different charges: NAD^+(-1), NADH(-2) and H ions(+1). A possible mechanism underlying this behaviour will be outlined in the discussion (Chapter 8).

5.5 DETERMINATIONS OF IMMOBILISED HLADH STABILITY

HLADH was immobilised to 3, 0.22 μm membranes as described in Section 3.3.2, except that for each membrane a different pH buffer was used. These were pH 8.0, pH 9.5, and pH 11.0, 0.1 M NaOH-glycine buffers. The same buffer was used for immobilisation, washing and the aqueous phase. Each day, for four days, the activity of each membrane was measured using the procedure outlined in Section 5.3. In between runs the membranes were blotted dry and stored in 10 ml of the appropriate buffer.

Figure 5.9 shows that at all pH values the immobilised enzyme preparations

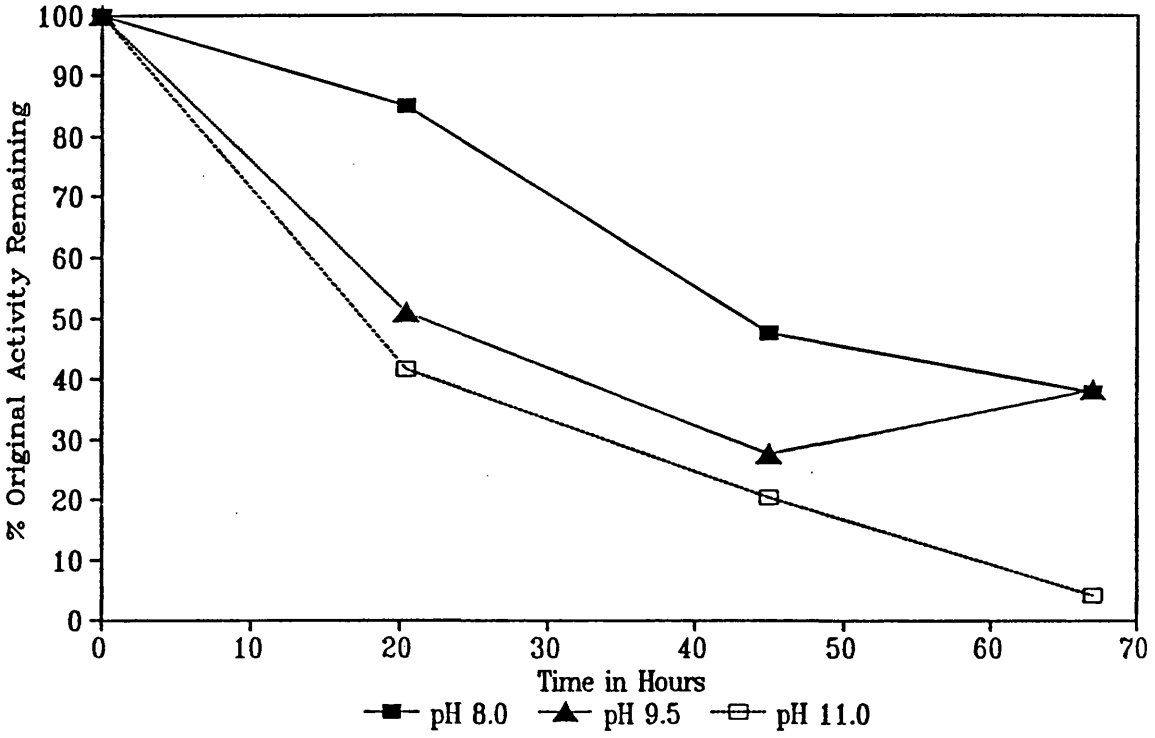


Fig 5.9. The Effect of pH on the Stability of Immobilised HLADH (the membranes were stored in the dark at room temperature– 18–20°C)

incurred substantial losses of stability. The highest stability observed at pH 8.0 was where approximately 45% of the original activity was retained. Over the range investigated the sequence of stability retention at different pH values, from the highest to the lowest, was pH 8.0 > 9.5 > 11.0.

Comparisons of stability between the soluble enzyme and immobilised HLADH are summarised in Table 5.1, results were expressed in terms of enzyme half-lives ($t_{1/2}$) ie. the time taken until 50% of the original activity was remaining. As actual activities between the two systems were markedly different the use $t_{1/2}$ allowed comparison of the stability trends to be made.

The results showed differences between the two systems with the soluble enzyme being the more stable form. This indicated that the immobilised enzyme environment was probably inducing an increased rate of enzyme denaturation. The exact mechanism was unknown but, given that during the experiment the enzyme-loaded membranes were held in buffer continually and only subjected to reaction conditions for approximately 5 minutes every 24 hours, it seemed likely that the reduction in stability was caused by an interaction of the membrane surface and enzyme. Disruption of the globular structure and spreading of protein molecules over support surfaces has been suggested as a mechanism for the irreversible loss of biological activity of adsorbed proteins and this may have occurred in this case (Trevan 1980).

The soluble enzyme showed optimum stability at pH 8.0 or below and the

Sample	Half Life of Enzyme in Hours		
	pH 8.0	pH 9.5	pH 11.0
Soluble Enzyme	66	80	14
Immobilised Enzyme	43	22	17

Table 5.1 **A Comparison of the Stability of Soluble and Immobilised Enzyme at Different pH Values**

immobilised enzyme showed optimum stability at pH 9.5. This suggested the environment of the immobilised enzyme had affected stability. The changes in stability were probably related to the ionic strength and pH effects outlined above and their interaction will be taken up in the discussion (Chapter 8).

5.6 SUMMARY

Examination of the kinetic behaviour of the immobilised enzyme using techniques similar to those employed for the soluble enzyme clearly showed systematic deviations from Michaelis-Menten kinetics and greatly reduced reaction rates than those for the soluble enzyme. Investigations to ascertain the cause of these effects implicated internal mass transfer limitation and microenvironmental effects as the key factors responsible for the observed deviation from Michaelis- Menten kinetics.

In the main, reduced specific reaction velocities probably reflected denaturation of a large proportion of the enzyme on immobilisation because none of the variations to the chemical environment enhanced reaction velocities to the order of magnitude observed in solution. Accelerated denaturation induced by immobilisation of the enzyme to the membrane support was also shown by a stability comparison with soluble enzyme.

Finally, the investigation revealed no unusual or novel properties of the

reaction system due to the presence of a two-phase system. All results were consistent with those obtained for enzymes immobilised in aqueous, single-phase systems. This gave some indication that the enzyme was supplied with octan-1-ol by diffusion from the organic phase rather than by direct interfacial contact and that deviations from Michaelis-Menten kinetics were probably largely the result of diffusional limitations within the membrane matrix.

CHAPTER 6

MEMBRANE CHARACTERISATION

6.1 INTRODUCTION

The experiments outlined in this Chapter were conducted to explore the possible influence of membrane structure on the results obtained in Chapter 5. The approach taken was to characterise more fully the physicochemical nature of the membrane and its influence on kinetic behaviour of the immobilised enzyme.

6.2 EFFECT OF MEMBRANE PORE SIZE ON INITIAL REACTION RATES OBTAINED USING IMMOBILISED HLADH

Four, different pore size, PVDF membranes were available. This presented the opportunity to investigate the effect on reaction rates of variation of a physical characteristic of the immobilised enzyme support.

Similar investigations of pore length on immobilised enzyme kinetics revealed that pore geometry affected the rate of transfer of substrate to enzyme and so was capable of influencing observed reaction rates (Goldstein 1976). So, investigation of the effects of pore size on reaction rate allowed further characterisation of enzyme kinetic behaviour.

Duplicate, 63 mm diameter circles of PVDF Minitan® membranes with nominal pore sizes of 0.1, 0.22, 0.45 and 0.65 μm were used. These were prepared and HLADH was immobilised onto them as described in Sections 3.3.1. and 3.3.2. The reaction conditions and procedures were the same as those described in Section 5.3, except that the pH was kept constant for all runs at pH 9.5.

The results are shown in Figure 6.1 where it can be seen that both reaction rate and the enzyme loading increased with pore size over the range used. Increase of enzyme loading with pore size indicated that loading occurred under conditions of mass transfer limitation; as pore size increased enzyme access increased and consequentially more was loaded in the 30 minute loading period. This shows that the membranes used in the previous Chapters were: a) loaded below their total capacity and; b) probably most of the enzyme was loaded on the membrane surface and areas most proximal to the surface. Interestingly this type of loading regime tends to reduce internal mass transfer limitation of immobilised enzymes. This is because the enzyme is loaded in a cross sectional profile similar to that of substrate and product profiles in an internally mass transfer limited system (Buchholz 1982). Consequentially any internal mass transfer limitations were probably less severe than would be possible with a more even internal distribution of the enzyme.

The increase of specific reaction rate with membrane pore size (a physical parameter) demonstrated clearly the presence of internal mass transfer limitation of the reaction velocity (recall, from Chapter 3, that external mass transfer limitation of the reaction velocity was rendered negligible by the selection of suitable stirrer

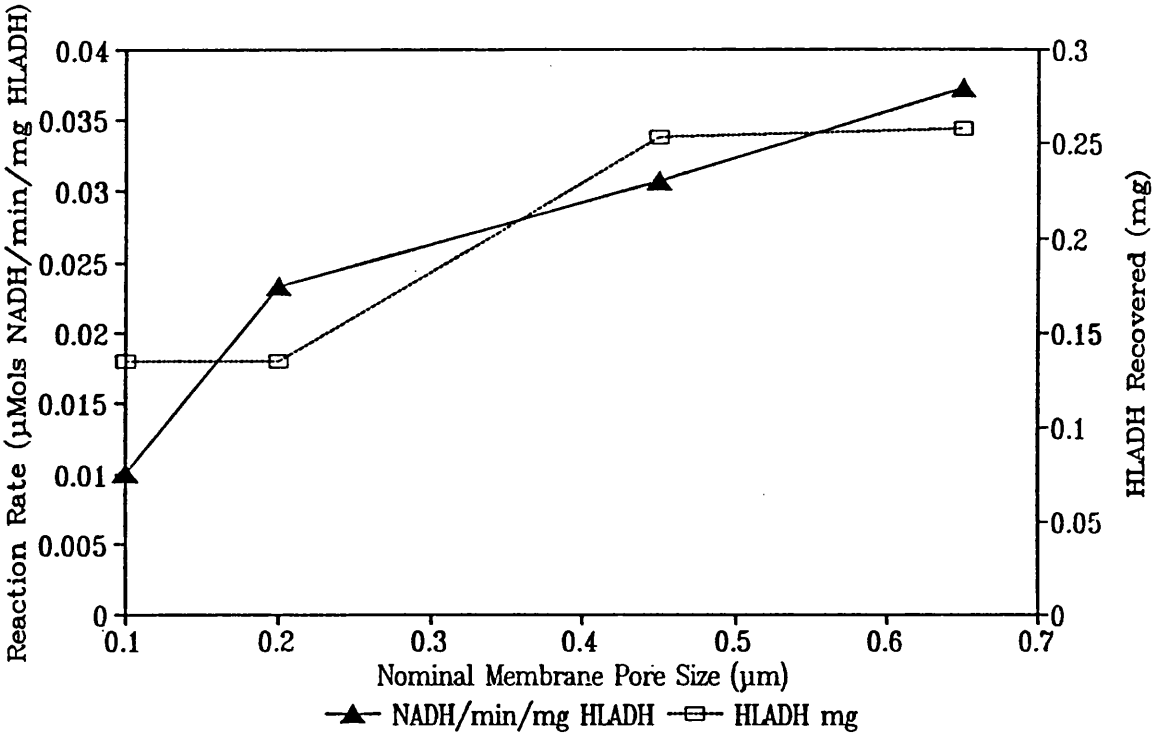


Fig 6.1. The Effect of Membrane Pore Size on the Activity of the Immobilised HLADH

speeds). In fact, as no stable reaction velocity was reached with increase in pore size then the reaction system must have been subject to a degree of internal mass transfer limitation at all the pore sizes used.

The situation can be described by the following mathematical expression for the effective substrate diffusion coefficient $D_e s$:

$$D_e S = D_s 0 . \frac{\epsilon}{\tau} . \frac{K_p}{K_r}$$

where: $D_s 0$= substrate diffusivity in bulk liquid; ϵ= membrane porosity; τ= membrane tortuosity factor (related to pore size) and; K_p/K_r= restricted pore diffusion parameter (a measure of the relative substrate and pore radii) (Keurentjes et.al. 1990). A similar expression would be valid for product diffusion.

From the equation above, an increase in tortuosity leads to a reduction of $D_e s$, ie. poorer substrate (or product) transfer rates within the membrane. In the membrane immobilised enzyme system the consequence of this was limitation of the reaction velocity by diffusion rates of a substrate or inhibition of enzyme activity by product diffusion rates (see Figure 6.1 which shows increase in the specific reaction rate with pore size).

Importantly, this experiment independently verified the influence of internal mass transfer limitations on reaction rate as suggested in Chapter 5.

6.3 ELECTRON MICROSCOPY OF THE MEMBRANES

This experiment was conducted as a visual aid to support the investigation of the effect of pore size outlined above.

Samples of the 0.1, 0.22, 0.45 and 0.65 μm membranes were taken and blotted dry. These were then coated with gold in an Edwards 150B sputter coater for 6 minutes at 1.5 KV, 60 mA and 0.15 mbar vacuum. Two samples of each membrane were used so that the original "permeate" and "retentate" sides of each membrane could be viewed.

Following preparation the samples were viewed using a Jeol JSM T330 scanning electron microscope. The accelerating voltage was 15KV. Photomicrographs were taken of the membrane structures resolved at 2000 times magnification.

Figures 6.2 to 6.5 show the structures of the 0.1, 0.22, 0.45 and 0.6μm membranes resolved at 2000 times magnification. Both sides of the membranes provided almost identical micrographs so only one set is shown. The micrographs clearly show the "sponge-type" structure of the membranes and the presence of tortuous pores. As pore size increased pores became apparently less tortuous and the membrane structure more open. This implied increased rates of both substrate and product transfer with pore size and the increase in specific reaction velocities previously observed.

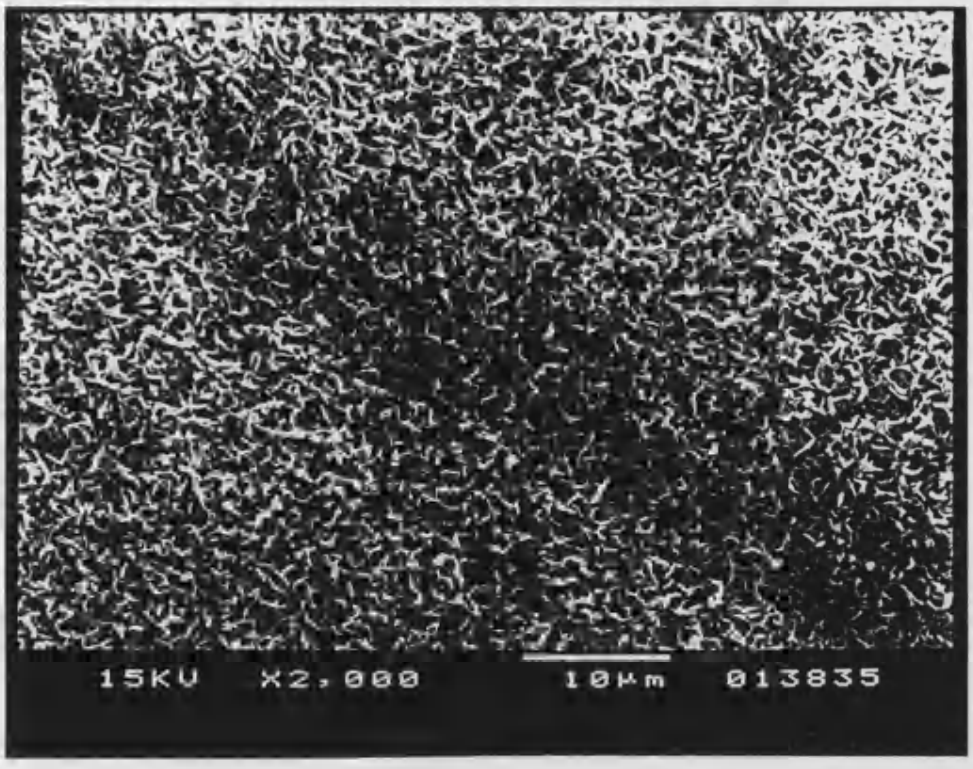


Figure 6.2 Photomicrograph of a 0.1 μm Membrane X2000

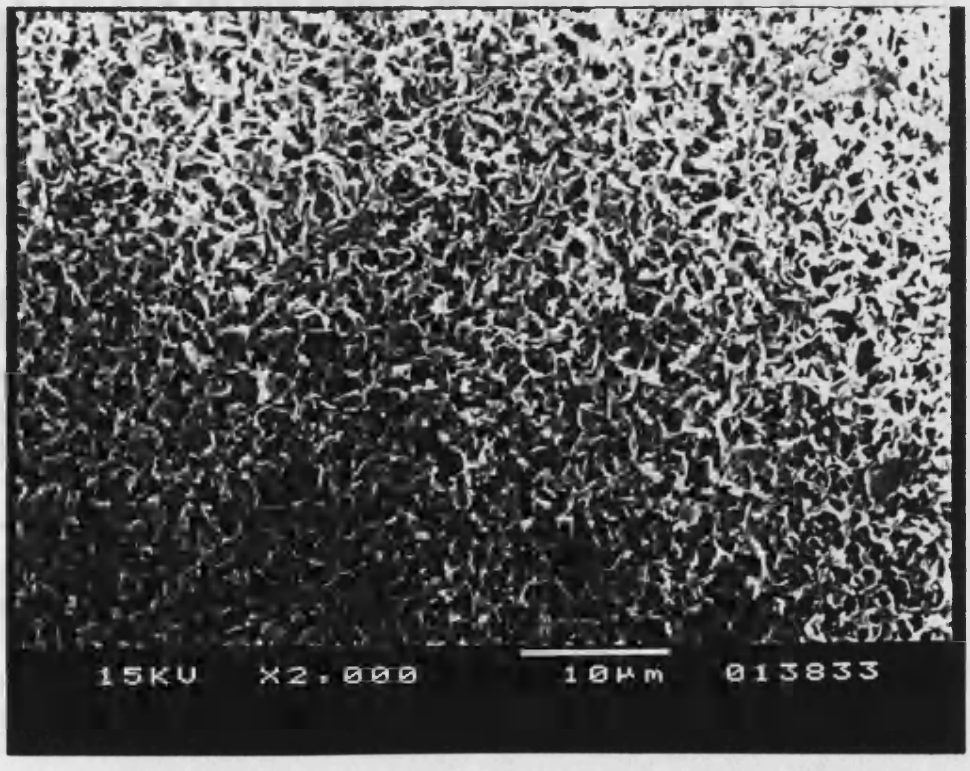


Figure 6.3 Photomicrograph of a 0.22 μm Membrane X2000

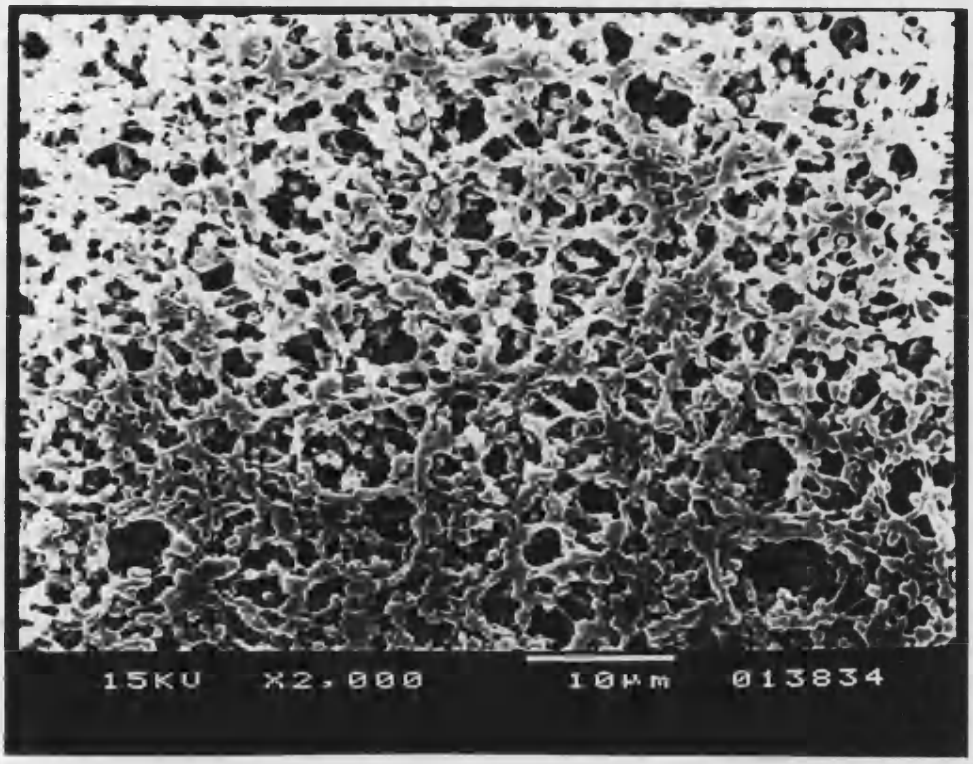


Figure 6.4 Photomicrograph of a 0.45 μm Membrane X2000

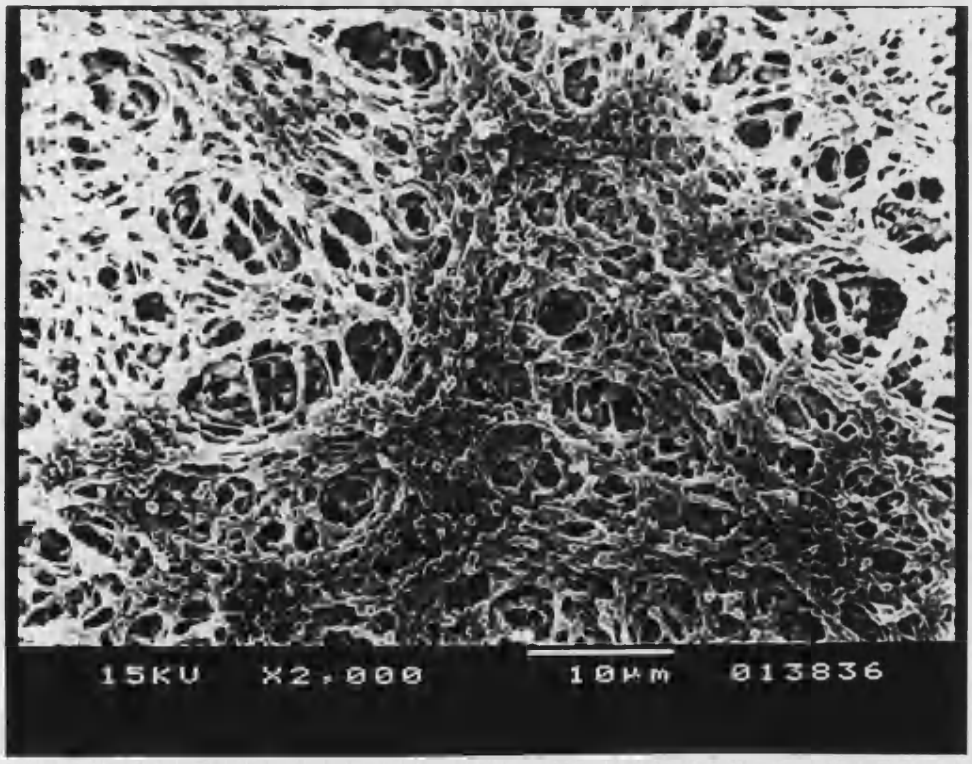


Figure 6.5 Photomicrograph of a 0.65 μm Membrane X2000

As a final point, it can been seen that there was a difference in the form of the membranes between the 0.1 and 0.22 μm membranes and the 0.45 and 0.65 μm membranes. This may have produced the two plateaus seen in the graph of recovered enzyme versus pore size and may reflect some differences in membrane chemistry between the 0.1/ 0.22 μm membranes and the 0.45/0.65 μm membranes.

6.4 TITRATION OF A 0.22 μm MEMBRANE

Given the results of variation of the aqueous phase pH and ionic strength (summarised in Chapter 5) it was important to attempt to understand the contribution of the membrane surface electrochemistry to these effects. For this reason the membrane was titrated to examine its surface charge characteristics.

A single, clean, membrane circle was taken (which contained no adsorbed protein) and finely shredded into approximately 1 mm wide strips. A volume of distilled water was taken and the pH adjusted to 7.0 using 0.01 M NaOH. The pH of the water was measured accurately. The shreds of membrane were blotted dry and placed in 25 ml of the distilled water. The membrane suspension was then titrated using standardised 0.01 M HCl. The titration was followed using a pH probe. After each addition of approximately 0.02 ml of acid the solution pH was allowed to equilibrate and the new pH noted. On completion the membrane was washed several times using the distilled water and the solution vacuum filtered to leave the membrane dry. The membrane shreds were then placed in another 25 ml of the distilled water

and titrated again using standardised 0.01 M NaOH. The procedure was repeated for the distilled water alone.

The acid and base titration curves for a 0.22μm membrane are shown as Figures 6.6 and 6.7 respectively. It can be seen the membrane displayed buffering capacity in both titrations. Additionally, in the base titration, the presence of buffering capacity at pH 9.5 indicated that at this pH (the pH at which the majority of immobilisation and reaction work was carried out) the membrane had positively charged groups available for neutralisation with NaOH. The membrane was constructed of polyvinylidene difluoride which is an uncharged polymer ($-CH_2CF_2-$) so this material itself could not have produced the observed charge. However, as PVDF is manufactured by free radical polymerisation it was likely that a number of initiation and termination groups remained on the membrane surface and so it was likely these compounds produced the observed membrane charge. In experiments with a very similar polymer, polytetrafluoroethylene (PTFE, $-CF_2-$), Bee et.al. (1983) estimated that 2% of the surface of PTFE latex beads was occupied by carboxyl compounds originally included in the bead manufacture as polymerisation initation and termination compounds. A net positive charge on the beads was attributed to these carboxyl compounds.

From the observation of a positive membrane charge it was noted that because the reaction buffer was above the isoelectric point of the enzyme (PI= 6.8 Sund and Theorell 1963) then the enzyme would have been negatively charged. This implied the immobilisation process was partly electrostatic in nature. Further, the observation

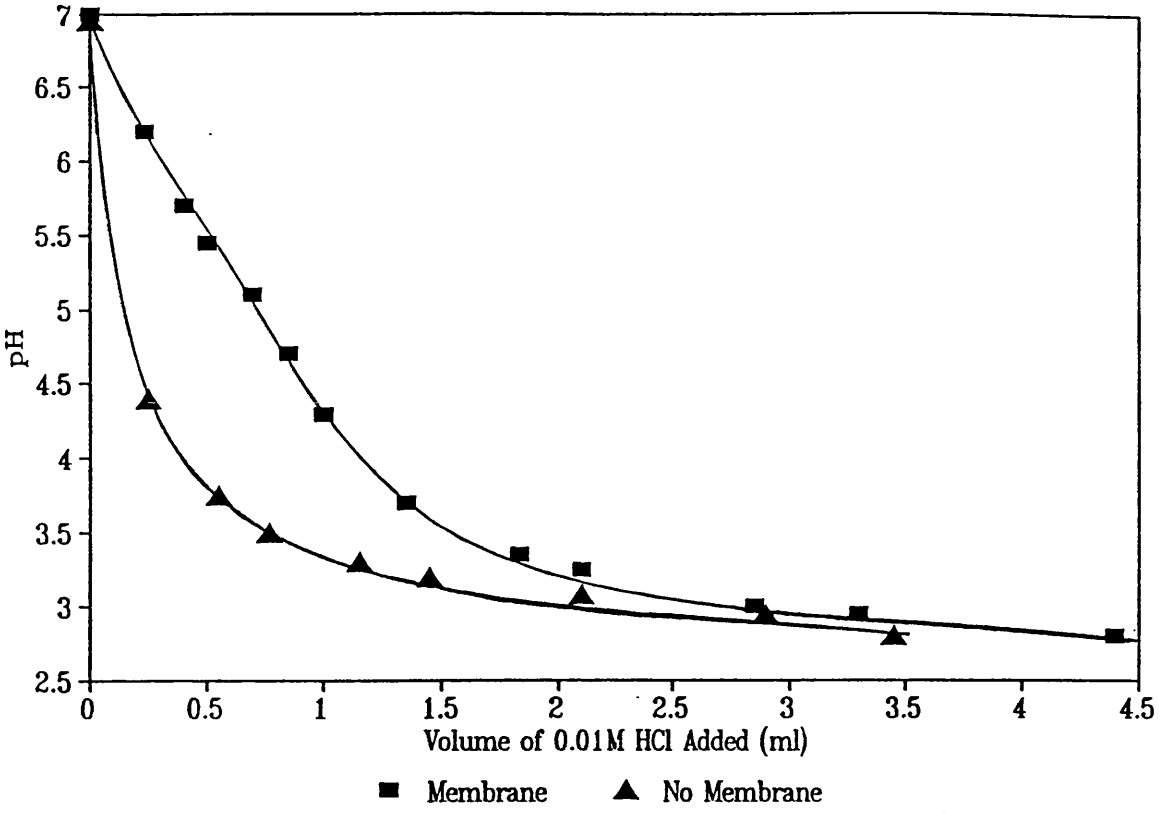


Fig 6.6. Acid Titration of a 63 mm Diameter, 0.22µm PVDF Minitan Membrane Circle.

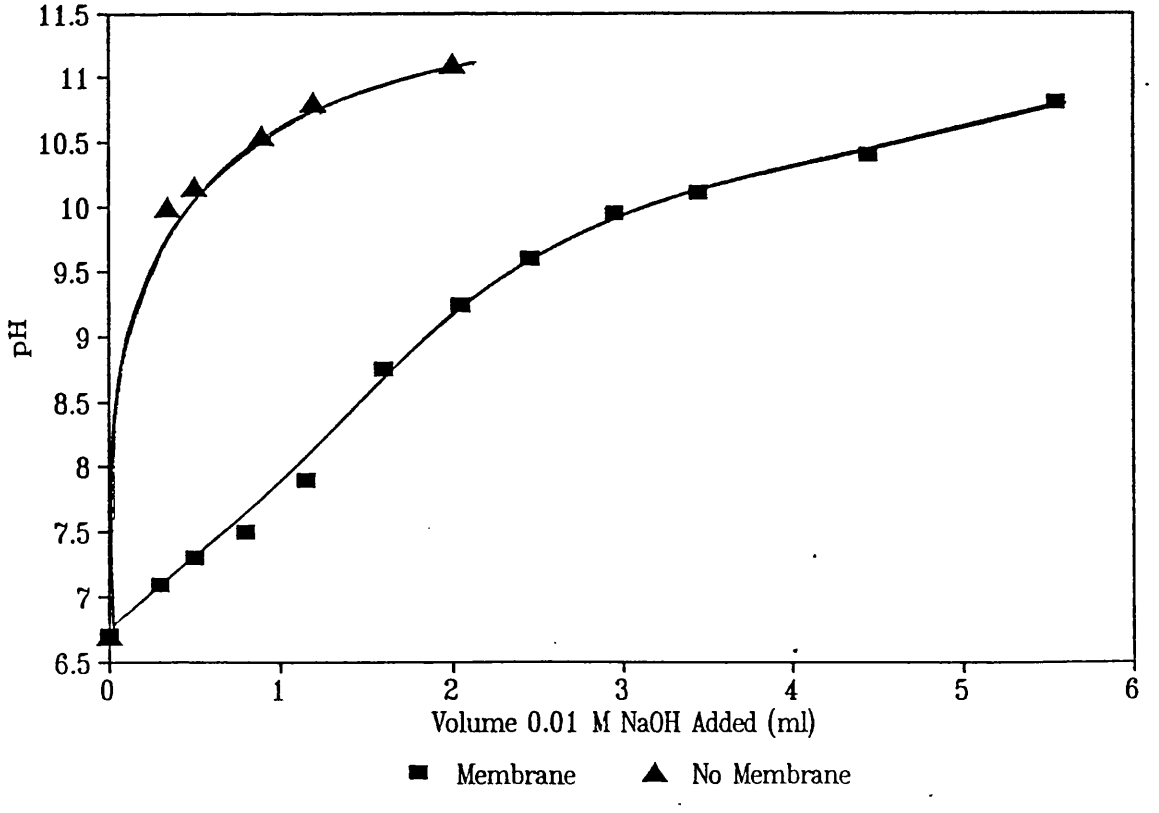


Fig 6.7. Base Titration of a 63 mm diamemter, 0.22µm Minitan membrane Circle.

of a net membrane charge suggested its importance in the determination of reaction rates, particular in the the pH and ionic strength effects discussed in Chapter 5.

6.5 A COMPARISON OF THE MASS TRANSFER OF OCTAN-1-OL AND THE INITIAL REACTION RATES OBTAINED USING IMMOBILISED HLADH

This experiment was included to provide an idea of the concentration of the organic phase substrate (octan-1-ol) that was available to the immobilised enzyme and its mass transfer rate into the membrane. This was an important consideration as although internal mass transfer limitation had been identified as a substantial influence on the immobilised enzyme kinetics only the aqueous phase substrate and products had been noted as contributing to the effect. By obtaining an idea of the octan-1-ol mass transfer rate into the membrane then the relative contribution of this substrate to the internal mass transfer limitation effect could be more fully understood.

Two 0.22 μm membranes were prepared and loaded with enzyme as outlined in Sections 3.3.1 and 3.3.2. Each was initially subject to reaction with 18 ml, 1% octan-1-ol (v/v) in 2,2,4-trimethyl pentane as an organic phase and 1 mg/ml NAD^+ in 15 ml, 0.1 M NaOH-glycine buffer pH 9.5 as an aqueous phase. The increase in absorbance at 340 nm was monitored for 5 minutes. The reactions were run in duplicate with washing of the respective compartments with buffer and solvent between each run. Additionally, each experiment was repeated using an aqueous

phase containing no NAD^+. A sample of the aqueous phase was taken at the end of each of these runs. The aqueous and organic phase stirrer speeds were set at 350 and 330 R.P.M. respectively for all runs. All reactions were run at room temperature which was measured. Adsorbed protein was recovered as described in Section 3.3.3.

The samples of the aqueous phases obtained were analyzed quantitatively for octan-1-ol using a method derived from Mänsson et.al. (1978). The following mixture was placed in a cuvette:

2.7 ml.....0.1 M, pH 9.5 NaOH-glycine buffer (assay buffer) made 10 mM in semicarbazide hydrochloride

0.1 ml.....10 mg/ml NAD^+ in assay buffer

0.15 ml.....aqueous phase sample or octan-1-ol standard

0.05 ml.....1 mg/ml HLADH in assay buffer

3.00 ml Total volume

N.B. The semicarbazide was present to react with the octyl aldehyde formed and so produce the corresponding semicarbazone. This effectively prevented the back reaction from occurring and ensured the reaction proceeded to completion.

After 1 hour at room temperature the cuvette containing the highest octan-1-ol standard was placed in a Cecil CE 588 spectrophotometer and the absorbance change at 340 nm monitored for 5 minutes. This was done to check for completion of the reaction (as evidenced by no increase in absorbance). The other samples and standard

absorbances were then read. A standard curve was created and octan-1-ol concentrations of the aqueous phase samples were read off.

The standard curve for this assay is shown as Figure 6.8. The comparative results of reaction rate and mass transfer rate are shown below.

Mean μMoles Octan-1-ol transferred to the bulk aqueous phase/minute = 0.171

Mean μMoles NADH produced by reaction/minute = 0.0166

The mass transfer rate to the bulk aqueous phase proceeded approximately ten times faster than the reaction rate observed. Assuming no repulsive interactions between the support and octan-1-ol and negligible modification of the transfer rate due to the exclusion of NAD^+ from the aqueous phase, then the rate of transfer of the substrate to the enzyme would be at least the same as the rate of transfer to the aqueous phase. From this it can be tentatively concluded that, at this octan-1-ol concentration, mass transfer of octan-1-ol was unlikely to have contributed substantially to the internal mass transfer resistance observed.

6.6 SUMMARY

Overall the experiments in this Chapter added more evidence to support the ideas raised in Chapter 5.

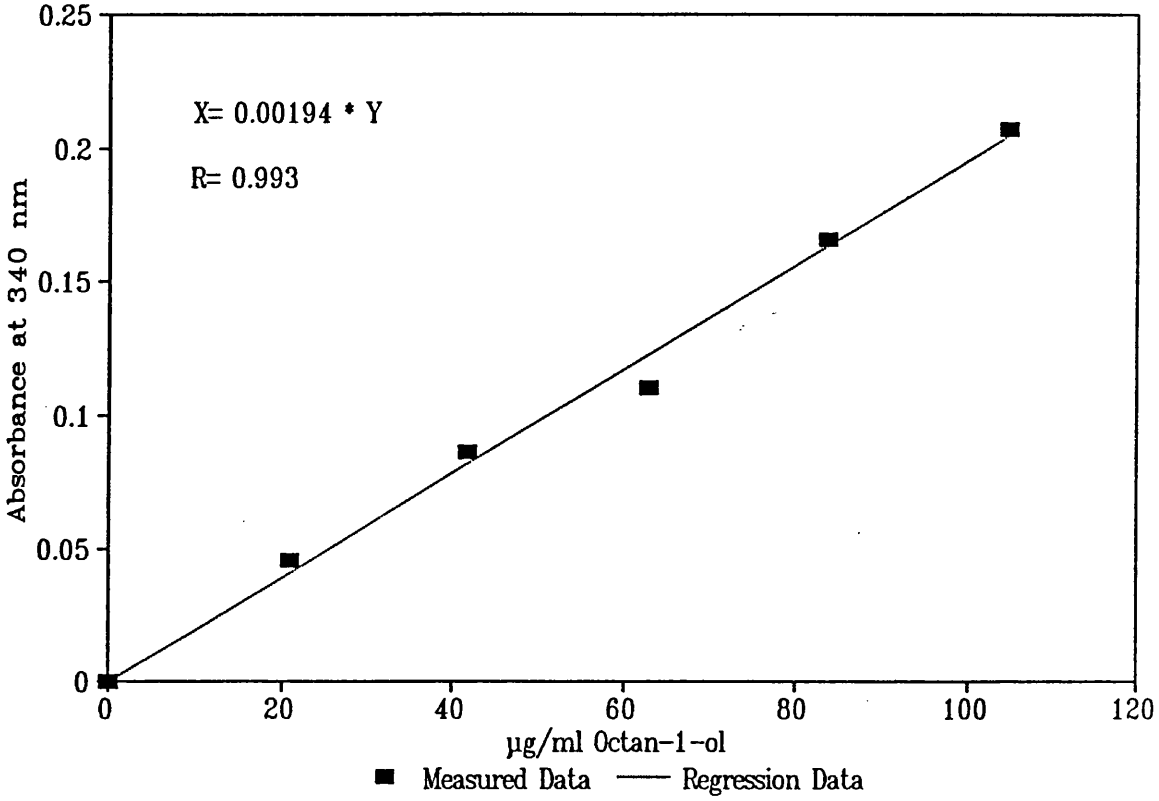


Fig 6.8. Standard Curve for the Assay of Octan-1-ol in the Aqueous Phase.

Increasing membrane pore size indicated the presence of internal mass transfer limitation of reaction velocities. This theme was visually reinforced by inspection of the electronmicrographs of the respective membranes.

The membrane titrations suggested the membrane to be positively charged supporting the idea of microenvironmental effects between the membrane and charged reaction substrates and products and suggesting electrostatic interactions to be part of the immobilisation mechanism.

Factors contributing to control of the reaction rate and their relative importances will be in reviewed in detail in the discussion (Chapter 8).

CHAPTER 7

DEVELOPMENT OF A LARGER-SCALE REACTOR INCORPORATING COENZYME REGENERATION

7.1 INTRODUCTION

Following analysis of the immobilised enzyme's kinetic behaviour the next development step was construction of and assessment of the feasibility of a two-phase membrane reactor incorporating coenzyme regeneration. In this Chapter the reaction system previously examined in the small glass reactor was transferred to a larger, test reactor based on a Millipore Minitan® unit (see Figure 7.1). This unit was chosen for a number of reasons including its solvent resistance and its ability to accept from 1 to 8 membrane plates allowing scale-up effects to be simply performed.

The initial objective of this work was to obtain a working reaction system in the Minitan® unit similar in nature to the system in the small glass reactor, before moving on to incorporate a coenzyme regeneration system to allow the use of less than stoichiometric amounts of coenzyme. As discussed in Chapter 1 this is usually essential for the economic operation of any large scale biotransformation system using a coenzyme dependant biocatalyst.

The approach taken was to keep the reaction system as similar as possible to the systems used previously, for example a 0.22 μm membrane was used as the

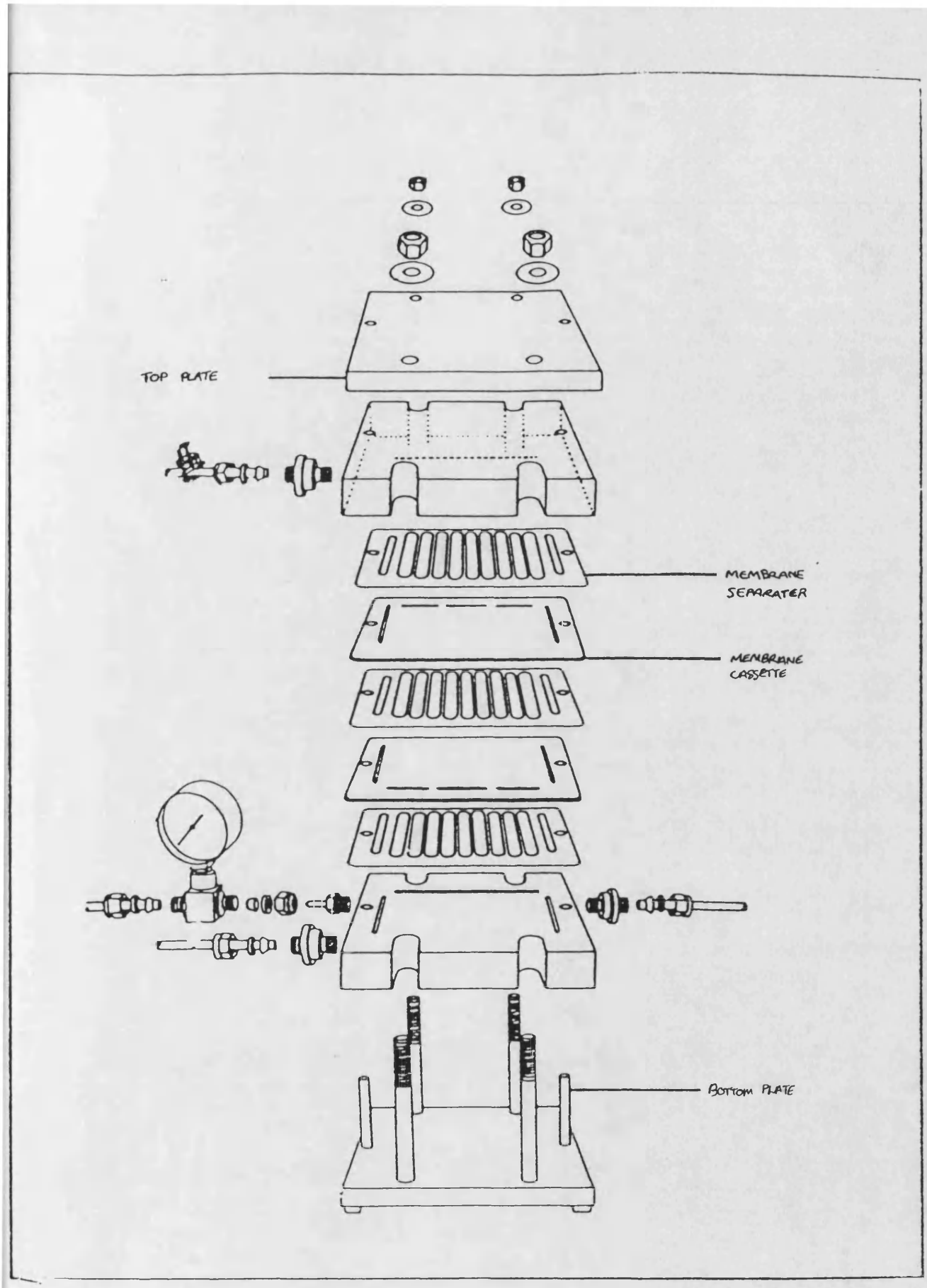


Figure 7.1 Exploded View of the Minitan® Unit

enzyme support. Although it was known from an experiment in Chapter 6 that the use of a larger pore size membrane increased reaction velocities, optimising the system was not the central issue. At this stage of development it was considered more important to choose the best understood reaction conditions which would allow the largest comparison of results between the small glass reactor and the new (at this point untested) Minitan® reactor.

7.2 ASSESSMENT OF THE SOLVENT RESISTANCE OF THE MINITAN® COMPONENTS

It was already known that both 2,2,4-trimethyl pentane and octan-1-ol caused no physically observable effects to the membrane itself. However, the solvent compatibility of the cassette support material and the silicone rubber membrane separators was unknown. Accordingly, samples of these materials were placed in octan-1-ol and 2,2,4-trimethyl pentane overnight. The following day the samples were examined closely against virgin samples for signs of physical change. In the case of the membrane separators considerable swelling had occurred in the sample retained in 2,2,4-trimethyl pentane. The cassette support material appeared unchanged. Hence solvent-resistant, 1 mm nitrile rubber sheet was obtained and new membrane separators were produced using the originals as a template. The rig was then constructed using the Minitan® unit as the test immobilised enzyme reactor.

7.3 PRELIMINARY EXPERIMENTS WITH THE MINITAN® UNIT

Experiments were carried out to examine the operational characteristics of the rig during enzyme immobilisation, start up and during runs. The rig was arranged as shown schematically in Figure 7.2.

A single, 0.22 μm membrane cassette was placed in the rig. 50 ml of 0.1 M, pH 9.5 NaOH-glycine buffer containing 38.5 mg HLADH was placed in the aqueous phase reservoir. For the immobilisation step the rig was operated as a dead end filter with full return of liquid crossing the membrane to the aqueous phase reservoir (see Figure 7.3). The immobilisation was allowed to run for 30 minutes at a flow rate of approximately 5 ml/min. The rig was then emptied and 100 ml of 0.1 M, pH 9.5 NaOH-glycine buffer placed in the aqueous phase reservoir and circulated at the same flow rate for 15 minutes. At the end of this period the reactor was drained and reconfigured to produce separate aqueous and organic phase circulation systems on either side of the membrane (see Figure 7.2). Both phases were fully recirculated and the system functioned as a batch reactor. 100 ml of 20% (v/v) octan-1-ol in 2,2,4-trimethyl pentane was then added to the organic phase reservoir and 100 ml 0.1 M, pH 9.5 NaOH-glycine buffer was added to the aqueous phase reservoir. Recirculation was then begun at a flow rate of approximately 5 ml/min for both phases. After a period of 5 minutes 23 mg of solid NAD^+ was added to the aqueous phase reservoir. The change in absorbance at 340 nm was monitored over time.

During the initial period of the run a small amount of phase mixing was noted;

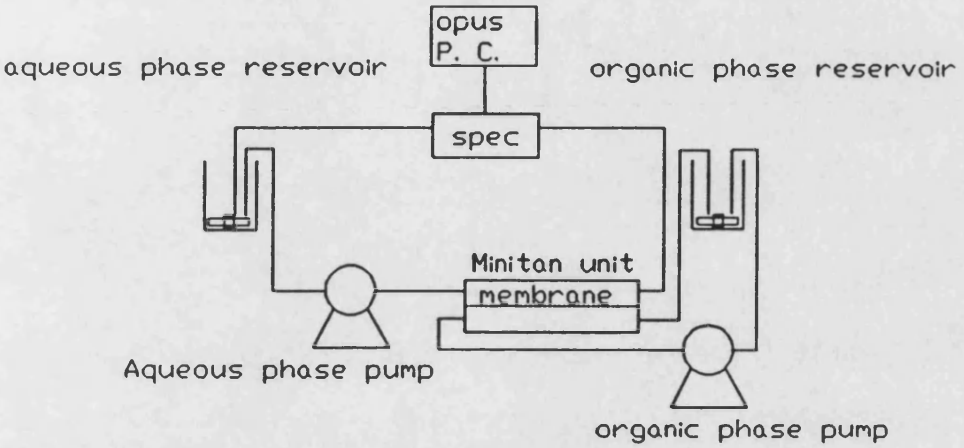


Fig 7.2 A Schematic Diagram of the Minitan Reactor and Ancillary Equipment

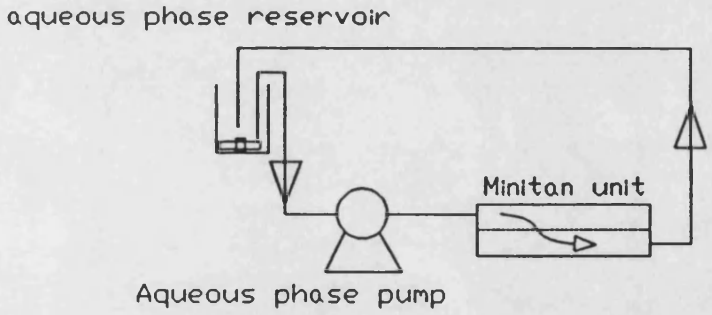


Fig 7.3 Schematic Diagram of the Enzyme Reactor Operating in Dead End Filtration Mode for Enzyme Immobilisation

the aqueous phase was permeating through the membrane and entering the organic phase reservoir. This was simply cured by applying a slight restriction to the organic phase reservoir return line. This produced a slight positive transmembrane pressure (<1 psi) on the organic phase. By positioning the organic phase reservoir feed pipe at the bottom of the reservoir the denser, invasive, aqueous phase portion was fed back into the reactor through the organic phase feed pipe and the applied transmembrane pressure caused this material to migrate across the membrane (presumably as droplets) back to the bulk aqueous phase. Within a maximum of 30 minutes from the reactor start up both phases were circulating separately. Hoq et.al. (1985a, 1985b) observed a similar effect and suggested it was a function of membrane hydrophobicity.

At 1 hour after start-up 5 μl of acetaldehyde was added to the aqueous phase reservoir. This was done to assess the potential of this compound to regenerate NAD^+ from NADH in this system as acetaldehyde had been reported as an effective coenzyme regeneration system in similar circumstances (Chenault and Whitesides 1987). The complete procedure was carried out at room temperature (18°C). The amount of HLADH immobilised was not determined. Figure 7.4 shows the monitored absorbance at 340 nm of the aqueous phase. The graph consists of several distinct regions.

It can be considered that octan-1-ol was being transferred to the aqueous phase before initiation of the reaction by addition of the NAD^+. This produced no change in absorbance at 340 nm but probably provided a substantial pool of substrate for

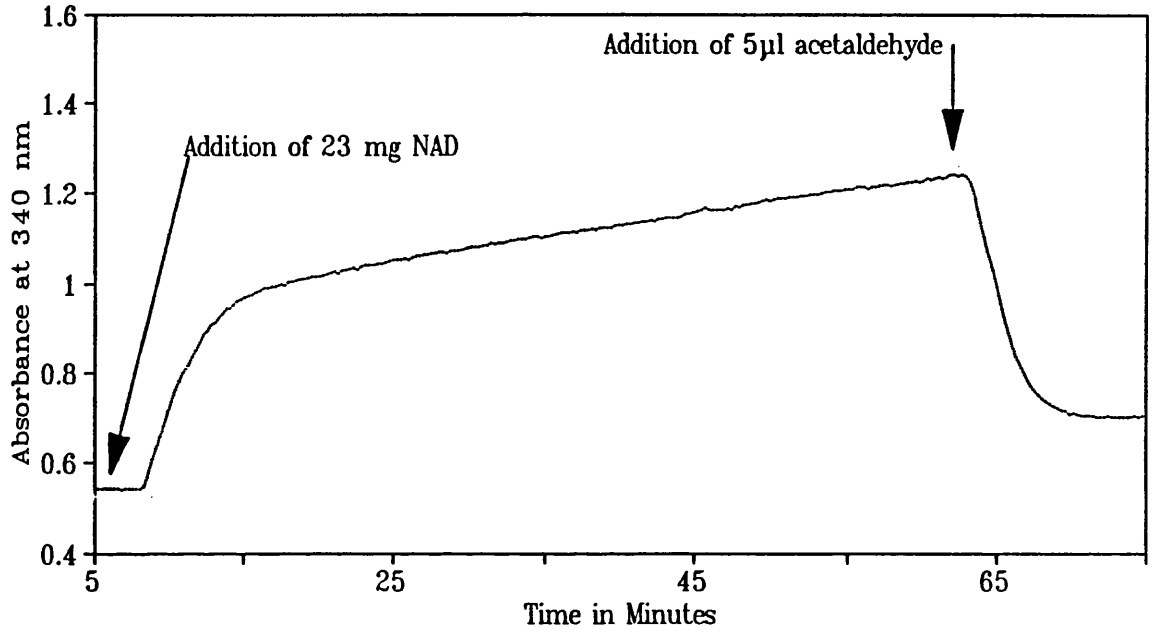


Fig 7.4. Preliminary Experiment in the Minitan Unit. Conditions: 1, 0.22µm plate; aqueous phase– 100 ml 0.1 M, pH 9.5 NaOH–glycine buffer (NAD and acetaldehyde added where indicated); organic phase– 100ml 2, 2, 4– trimethyl pentane containing 20% (v/v) octan–1–ol; Temperature 17°C.

immediate reaction. Upon addition of NAD^+ reaction proceeded rapidly utilising the pool of octan-1-ol available. As no NAD^+ wash was carried out during the immobilisation stage the addition of solid NAD^+ into the aqueous phase reservoir probably caused desorption of active enzyme which functioned to enhance the reaction rate and contribute to the steep increase in absorbance.

Rapidly, this substrate pool was utilised and then reaction proceeded at a constant rate until shortly after the addition of the acetaldehyde. This caused regeneration of the accumulated NADH to NAD^+ via HLADH. This step occurred rapidly. Following this period was another period of stable absorbance indicating a dynamic equilibrium between NAD^+ and NADH.

Hence, results demonstrated the feasibility of the Minitan® unit as a two-phase reactor which could accomplish coenzyme regeneration.

7.4 PROTEIN RECOVERIES

The amount of enzyme immobilised was determined using the SDS wash technique outlined in Section 3.3.3. The only modification was the use of larger volumes of the SDS solution to facilitate washing of the Minitan® cassettes. Volumes used ranged from 70 to 200 ml depending on the number of membrane plates washed. Protein recovery results for experiments in the Minitan reactor are summarised in Table 7.1. Reaction rates can be considered as semi-quantitative as some results were

Experiment reference	Number of Minitan® Plates	Total HLADH Recovered (mg)	HLADH Recovered ($\mu g/cm^2$)	Specific Reaction Rate µMoles NADH or Octyl Aldehyde/min/mg HLADH
Reactor productivity, Section 7.6	1	2.33	38.8	0.020
Aborted batch experiment, Section 7.7.2	3	9.33	51.8	-
Regeneration experiment using HLADH, Section 7.7.3	5	18.00	60.0	0.019
Regeneration experiment using YADH, Section 7.7.3	5	10.67	35.6	0.026

Table 7.1 Protein Recoveries for Experiments in the Minitan® Reactor

obtained from 24 hour batch reactions where the specific reaction rate was derived by dividing the final yield of octyl aldehyde by the time period of the reaction.

From the results it can be seen that the amounts of protein recovered were consistently much higher than those from the small glass reactor (compare these results with those in Table 3.1). This was probably because of differences in the loading regimes. This point is discussed further in Section 7.6. Further, as in Table 3.1 (giving protein recoveries in the small glass reactor), protein recoveries varied disproportionately to the specific reaction rates, again suggesting mass transfer limitation of the reaction.

7.5 PUMP CALIBRATIONS

The Eyela MP-3 pumps (Rikakikai Ltd) were calibrated by measuring the volume of liquid displaced in one minute at several different settings. Each measurement was repeated 3 times for each setting to produce an average estimate of the true flow rate. This data was used to produce a linear regression correlation of flow rate against pump setting. A typical calibration obtained is shown as Figure 7.5. From this graph it can be seen that flow rate increased linearly with pump speed over the range tested and consequently flow rates were easily controllable.

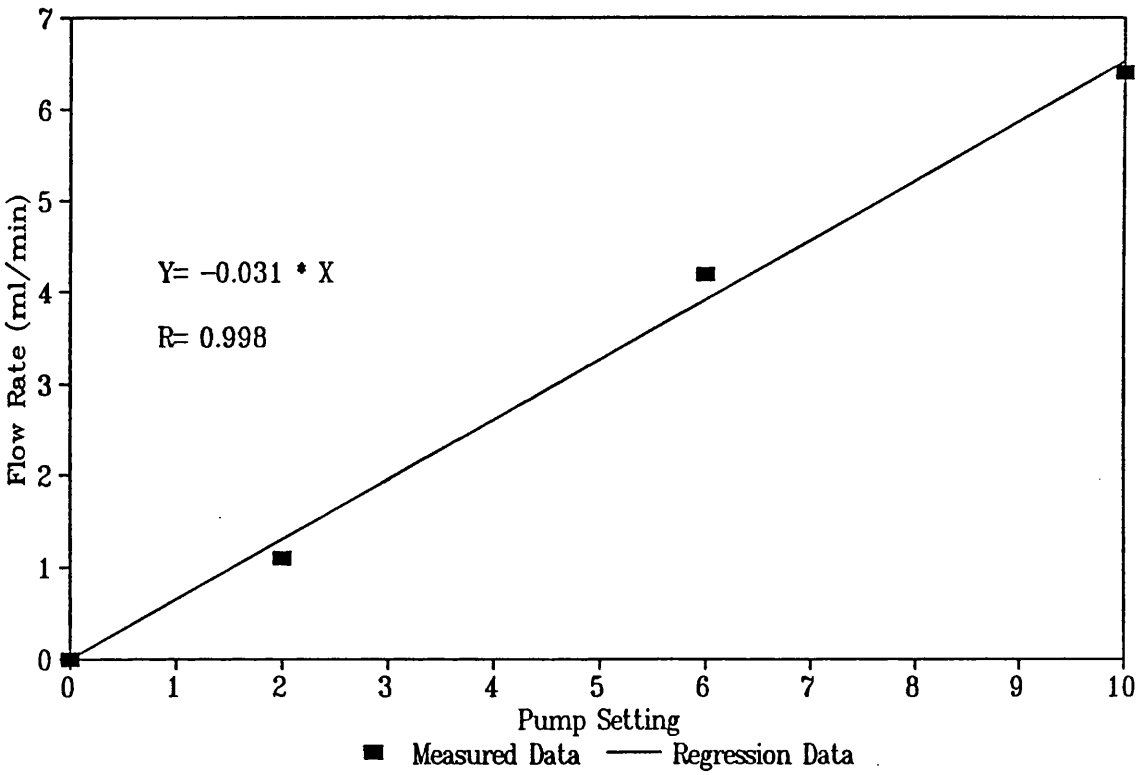


Fig 7.5. Sample Calibration of Eyela MP-3 Pump

7.6 EFFECT OF THE AQUEOUS AND ORGANIC PHASE FLOW RATES ON THE REACTOR PRODUCTIVITY

An earlier experiment using the small glass reactor had shown that the reaction velocity was related to the aqueous phase rheology (see Section 3.4.6). Essentially, in the small glass reactor, increasing the stirrer speed reduced the effect of external mass transfer limitation and so increased the reaction velocity. Importantly this effect was only observed with respect to the aqueous phase.

As the enzyme system was being assessed in a new reactor configuration the rheology of the system was likely to be different. So, the effect of the phase flow rates on the reaction velocity was investigated to assess the extent of any external mass transfer limitations.

A single, 0.22 μm membrane cassette (area 60 cm^2) was placed in the rig and 100 ml of 0.1 M, pH 9.5 NaOH-glycine buffer containing 100 mg HLADH was placed in the aqueous phase reservoir. The enzyme solution was used to load the membrane in dead end filter mode as described above in Section 7.2. Loading ran for 1 hour at 9.6 ml/min. The aqueous phase was then drained and the reservoir rinsed twice with buffer before 250 ml of the same buffer was added. This was recirculated in the rig for 15 minutes and then the procedure was repeated. The reactor was then drained and the aqueous phase reservoir rinsed twice with buffer. The reservoir was then loaded with 250 ml of the same buffer containing 250 mg NAD^+. This was recirculated through the reactor for 1 hour. At the end of this period the reactor was

drained and the reservoir rinsed with buffer. The reactor was then reconfigured to provide a single pass mode (see Figure 7.6). The reservoirs were appropriately loaded with 250 ml, 0.1 M, pH 9.5 NaOH buffer containing 250 mg NAD^+ and 250 ml 2,2,4-trimethyl pentane containing 1% (v/v) octan-1-ol.

Flow through the rig was begun and the aqueous and organic phase flow rates were adjusted to 1.0 ml/min and 6.0 ml/min respectively. Data logging was begun. When the change in absorbance appeared constant for 3 minutes the flow rates were changed to new values. The flow rates used were- 2.5, 4.0, and 6.0 ml/min for the organic phase against 1.0, 3.3, 5.7, 8.0 and 10.8 ml/min for the aqueous phase. All combinations of these flow rates were used, a new one being selected when a constant absorbance at 340 nm was obtained. The whole procedure was performed at room temperature (17°C). At the end of the experiment the amount of protein on the membrane was determined using the method outlined in Sections 3.3.3. and 7.4.

A plot of the effect of the phase flow rates on the reaction rate is shown as Figure 7.7. Reaction rates were calculated by multiplying the effluent NADH concentration by the effluent flow rate and dividing the product by the amount of HLADH recovered.

From the graph it can be seen that an increase in the aqueous phase flow rate caused an increase in the reactor productivity indicating the presence of external mass transfer limitation of the reaction rate with respect to the aqueous phase. Also it can be seen that for the aqueous phase, at all organic phase flow rates used, reaction

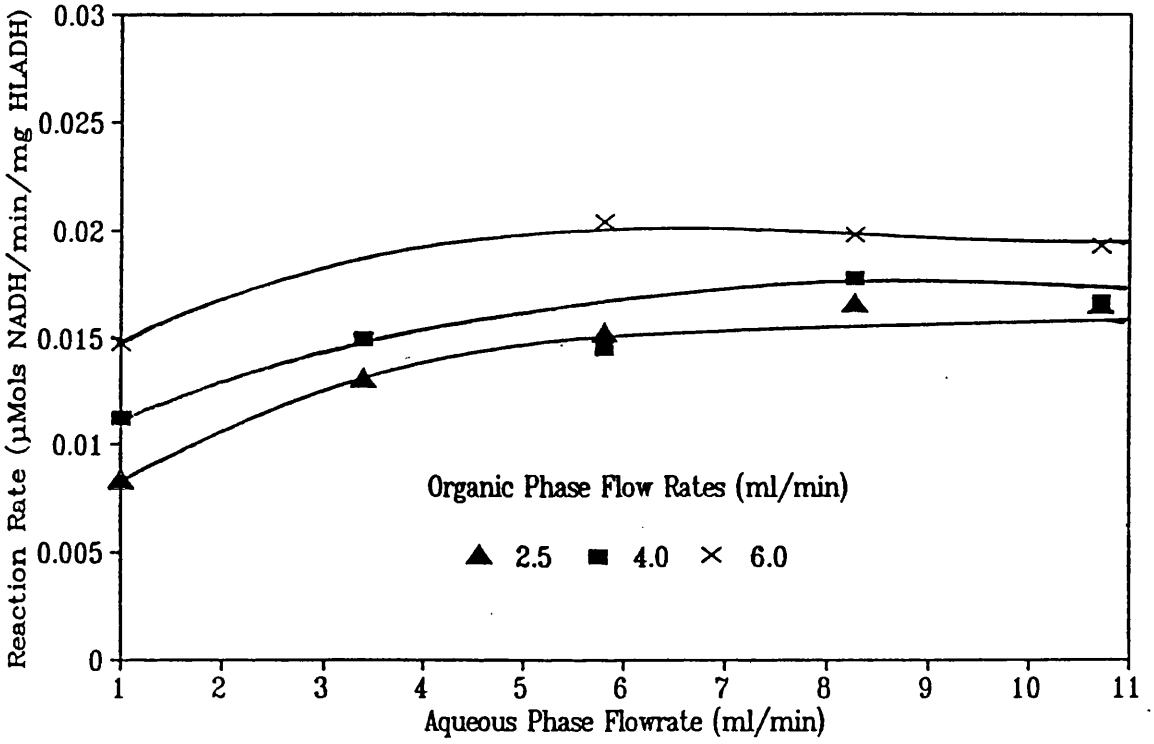


Fig 7.7. The Effect of Aqueous and Organic Phase Flow Rates on Reaction Rate.
(conditions were 1 mg/ml NAD, 1% Ooctan-1-ol, 1 reactor plate, 60 cm^2 area)

rates stabilised at approximately 6.0 ml/min. This indicated that external mass transfer limitation of reaction velocity became negligible above this value. This result paralleled the behaviour of the aqueous phase in the small glass reactor (see Section 3.4.6). Hence, in future experiments this flow rate was chosen for the aqueous phase.

The results also showed an increase in reaction rate with organic phase flow rate which demonstrated the presence of external mass transfer limitation with respect to the organic phase. In the small glass reactor external mass transfer limitation of the reaction rate was not detected for the organic phase.

It was expected that the Minitan® reactor, like the small glass reactor, would have shown no external mass transfer limitation with respect to the organic phase and so this result was surprising. The two principle differences between the small glass reactor and the Minitan® reactor were the method of fluid mixing and the method by which the enzyme was immobilised. It was considered the difference in response of the two systems to the flow regime of the organic phase lay with at least one of these factors.

It was observed earlier that the membranes used in the small glass reactor were loaded by diffusion under conditions of mass transfer limitation and it was suggested this resulted in the enzyme being immobilised in a profile across the membrane similar to the diffusion profile of a substrate or product (see Section 6.2). In the Minitan® reactor the enzyme was loaded by convection, the enzyme solution being forced through the membrane. This method of loading probably caused the

enzyme to be immobilised relatively uniformly within the membrane and so also lead to the immobilisation of much more enzyme; in this experiment 2.33mg of enzyme was recovered from one 60cm^2 membrane or a recovery of approximately 39μg/cm^2. This was approximately double the amount of enzyme loaded onto membranes used in the small glass reactor (see Table 3.1).

External mass transfer could be viewed as occurring prior to reaction (ie. external mass transfer was responsible for conveying substrate to the surface of the membrane) and internal mass transfer could be viewed as occurring simultaneous to reaction (ie. as substrate traverses the membrane it is used by reaction and so there is that much less substrate to be used for reaction deeper within the membrane).

By combining these effects it can be seen that in the small glass reactor substrate was passed to the surface of the membrane by a relatively large diffusion gradient (1% octan-1-ol in the bulk solution and 0% octan-1-ol within the membrane) and then partitioned into the aqueous phase within the membrane where it was reacted with enzyme probably laid down in a profile similar to that of the concentration profile of octan-1-ol within the membrane. Consequentially, as mixing of the organic phase was increased no increase in reaction rate was observed as the supply of octan-1-ol was probably met by diffusion alone.

In the Minitan® reactor the situation was similar but more enzyme was present which was likely to have been laid down uniformly across the membrane. In this case, the following may have occurred: octan-1-ol passed to the membrane surface

by diffusion and partitioned into the membrane would have been rapidly consumed by reaction leaving the enzyme at the centre of the membrane starved of substrate. Also, as the flow rate increased the concentration of octan-1-ol at the surface of the membrane probably increased and so the partition driving force would have increased with more octan-1-ol being available in the membrane for reaction. Hence, appreciable substrate concentrations would have reached the enzyme at the centre of the membrane and reaction rates increased.

Finally, it can also be seen from the graph (Figure 7.7) that external mass transfer limitation of the organic phase was not eliminated by the flow rates used. This reflected a practical consideration as the highest flow rates (for the aqueous and organic phases) were causing excessive backpressure and pulsing of flow. It was decided to err on the side of caution and not to damage the rig by using flow rates which in any case would not be sustained in longer term experiments. In future experiments a flow rate of 4.0 ml/min was used for the organic phase representing a compromise between sustainability of the equipment and minimisation of external mass transfer limitation.

7.7 BATCH EXPERIMENTS

Experiments were carried out using the reactor in batch mode (ie. full recirculation of both phases- see Figure 7.2) to examine the kinetics and equilibrium of product formation and to assess the effectiveness of coenzyme regeneration strategies.

7.7.1 Gas Liquid Chromatography (GLC)

A suitable technique was required to follow product formation in the batch experiments especially when the coenzyme regeneration system was used as the change in absorbance at 340 nm in the aqueous phase is no longer proportional to the reaction rate. This arises because the coenzyme regeneration system catalyses the conversion of the reaction product (NADH) back to NAD^+. So, following the reaction rate by measurement of the NADH concentration at 340 nm is not appropriate.

It was obviously most desirable to measure the accumulation of product (octyl aldehyde) directly. As both octan-1-ol and octyl aldehyde partition markedly into organic solvents then measurement of their concentrations in the organic phase would be a very close approximation of their total content (their log P values are 2.9 and 2.4 respectively, ie. their concentration in the aqueous phase will be only a few percent of their concentration in the organic phase). The regeneration reactant (ethanol) and product (acetaldehyde) were more difficult to devise a measurement system for as these tended to partition less selectively between the phases. It was decided for simplicity to assay their concentrations in the organic phase simultaneously with the octan-1-ol and octyl aldehyde concentrations.

Monick (1968) suggested GLC for the analysis of alcohols and carbonyl compounds. Hence a Porapak®P column was used in a Pye Unicam series 104 chromatograph fitted with a flame ionisation detector. The carrier gas was nitrogen

and the flow rate was 40 ml/minute. The oven, detector and injector temperatures were all maintained at 200°C. The chromatograph output was sent to an LDC Milton Roy C1-10B integrator and companion plotter.

A standard solution was made up in 2,2,4-trimethyl pentane of all components of interest; octan-1-ol, octyl aldehyde, ethanol and acetaldehyde. The components were added one by one and their respective retention times noted. All separated. Next an internal standard was selected. This was hexan-1-ol as its response factor was similar to that of the other compounds and also it did not lengthen the run time which was already approximately 25 minutes. For each component a standard curve was made up of concentration against peak height.

A typical chromatograph obtained using the Porapak P® column is shown as Figure 7.8. The trace demonstrates that separation of all components of interest was achieved.

Standard curves obtained for octan-1-ol, octyl aldehyde, ethanol and acetaldehyde are given as Figures 7.9 to 7.12. It is worth commenting that peak height was used as the relative measure of concentration rather than the usually favoured peak area. This was the case simply because the plots of peak height against concentration gave less curved responses than comparable plots of peak area. Hence, plots of peak height were more accurate as standard curves for the determination of unknown octan-1-ol and octyl aldehyde concentrations.

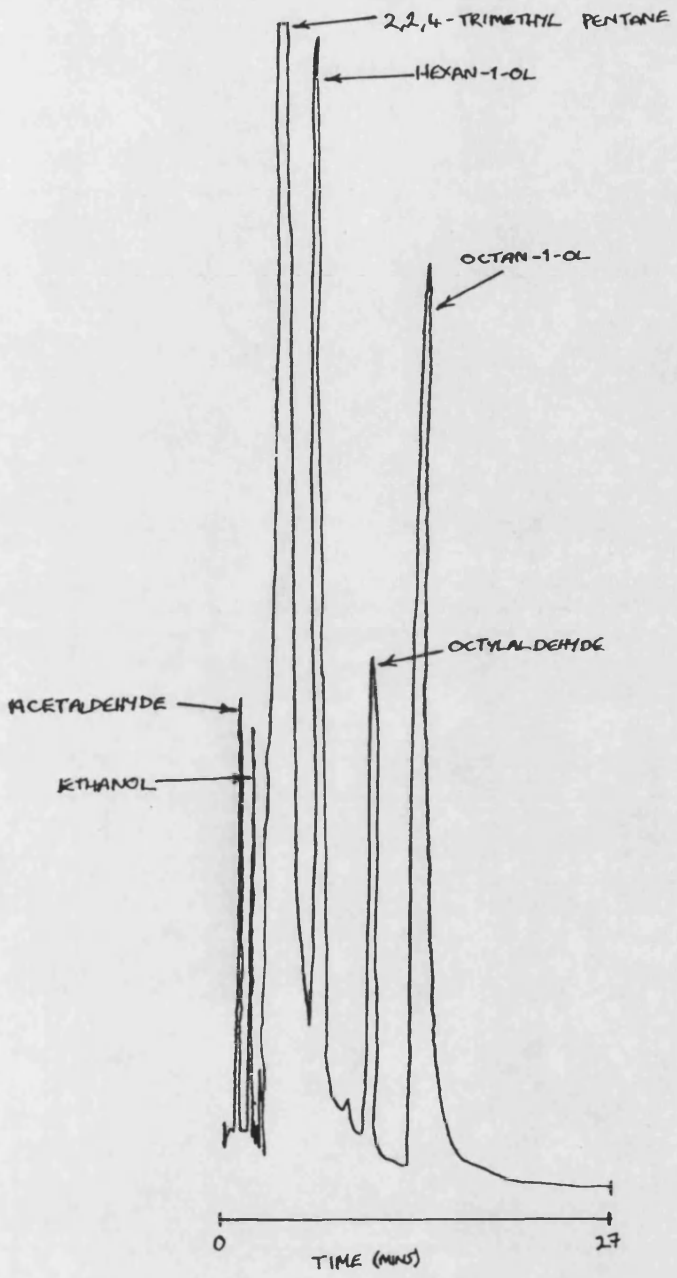


Figure 7.8 A Typical Trace Obtained using the Porapak® P GLC Column

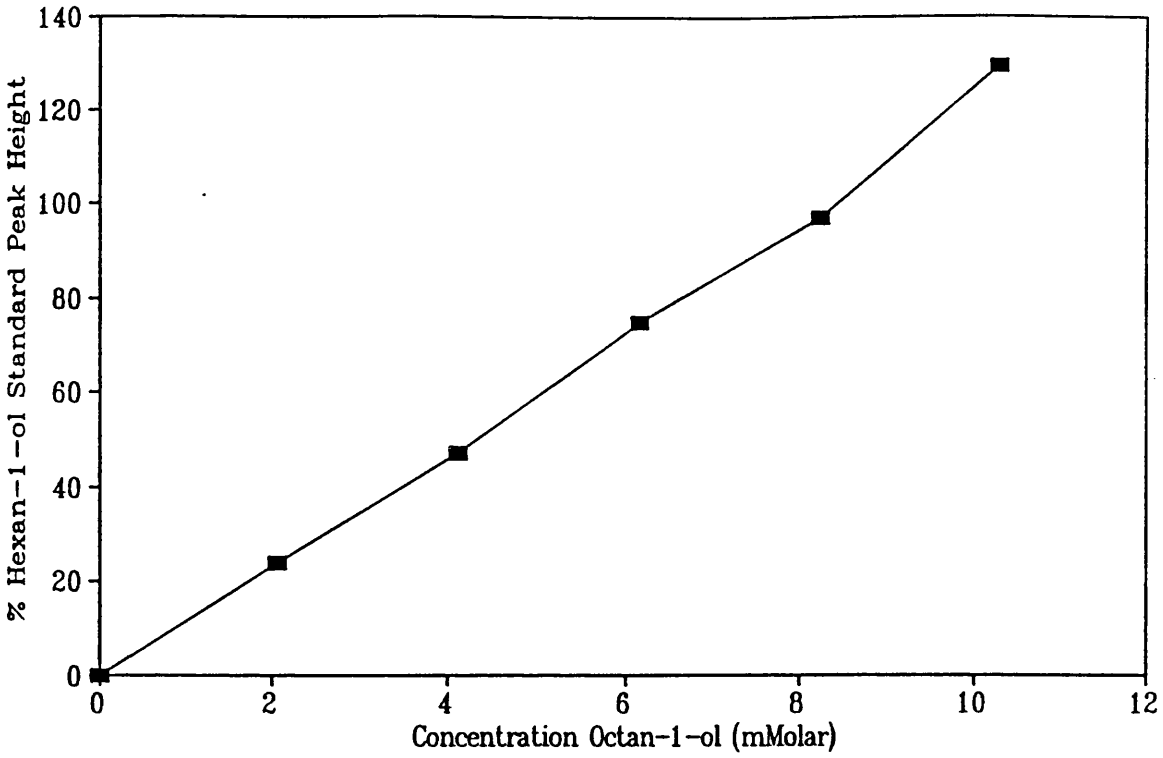


Fig 7.9. Octan-1-ol Standard Curve for GLC Analysis. (results are expressed as % octan-1-ol peak height / hexan-1-ol peak height)

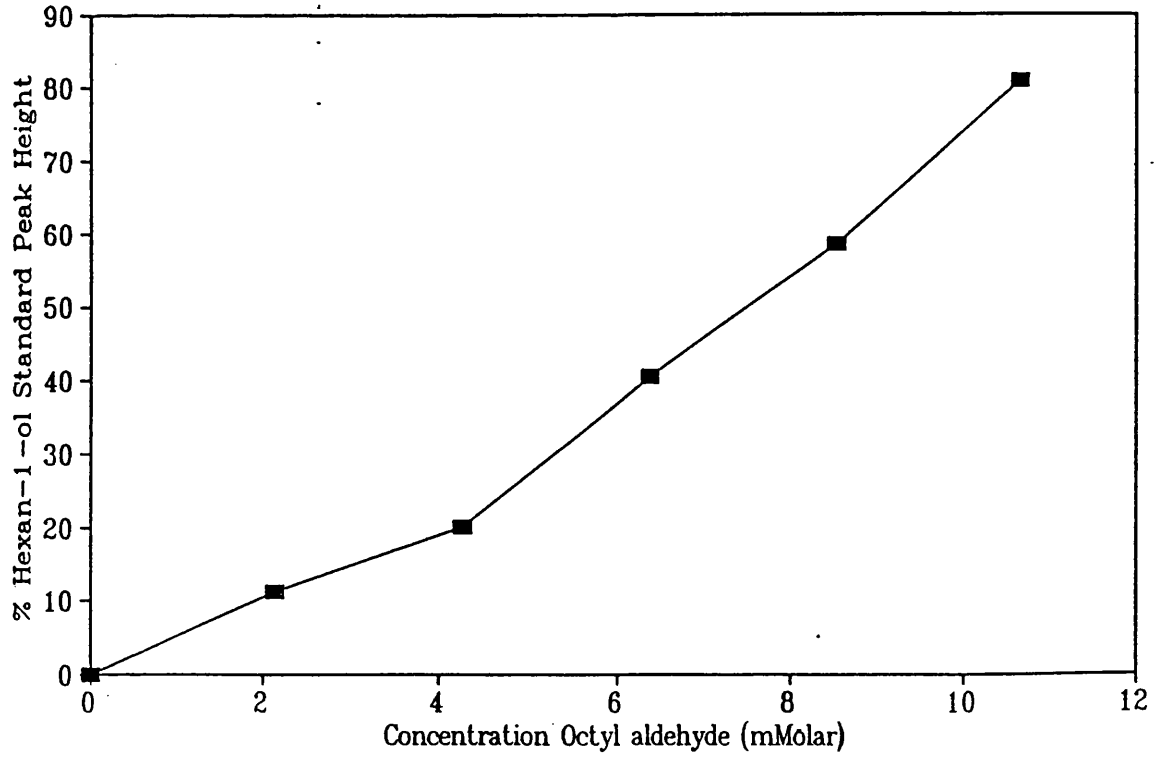


Fig 7.10. Octyl aldehyde Standard Curve for GLC Analysis. (results are expressed as % octyl aldehyde peak height / hexan-1-ol peak height)

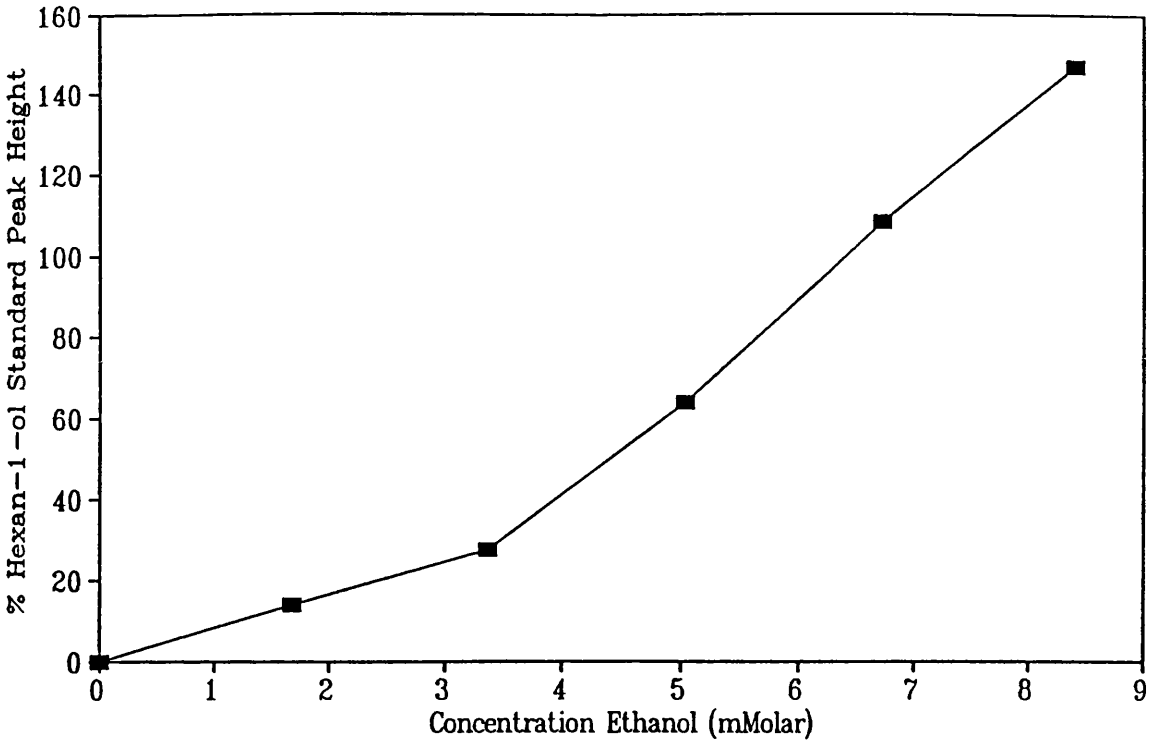


Fig 7.11. Ethanol Standard Curve for GLC Analysis (results are expressed as % Ethanol peak height / hexan-1-ol standard peak height)

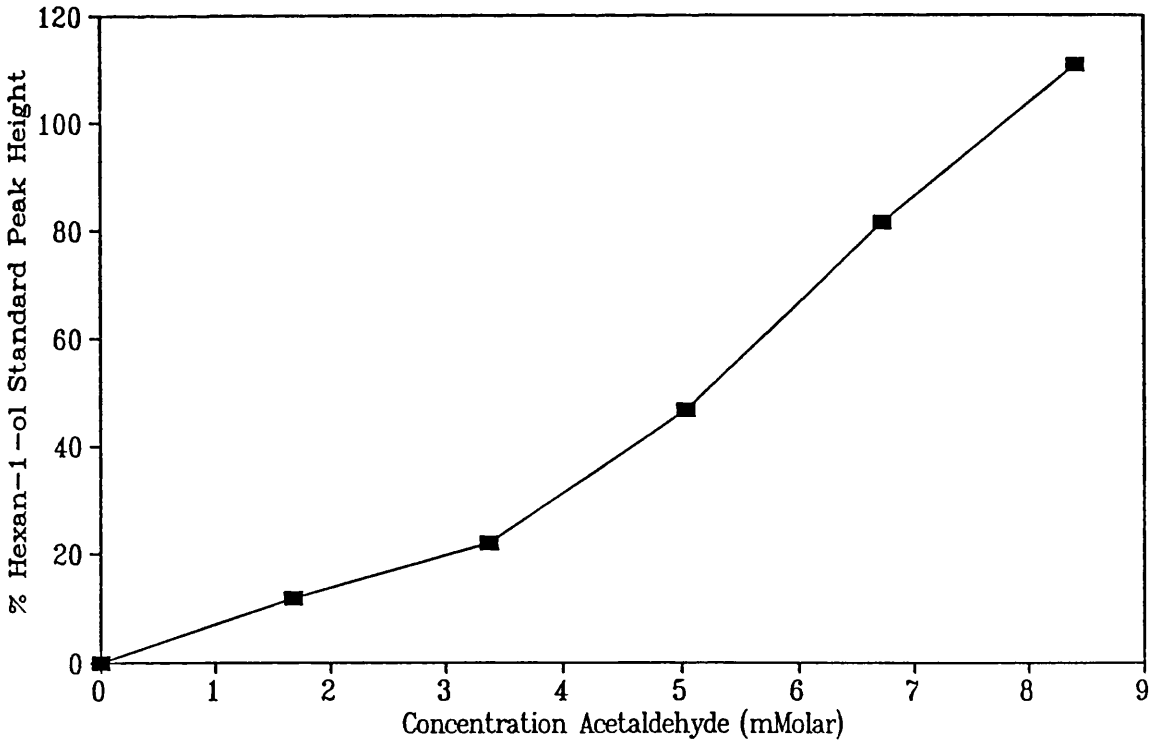


Fig 7.12. Acetaldehyde Standard Curve for GLC Analysis (results are expressed as % acetaldehyde peak height / hexan-1-ol standard peak height)

The reason for this lay with the fine tuning parameters of the integrator. Small changes in the separation and retention times of the internal standard peak (hexan-1-ol) and the octan-1-ol and octyl aldehyde peaks were prone to occur with each run. Although always clearly separated sometimes the octan-1-ol and octyl aldehyde peak were measured from the base line of the hexanol peak and sometimes from the "natural" base line for the separation. At low concentrations octan-1-ol and octyl aldehyde peak heights and areas tended to be measured from the hexan-1-ol base line. At high concentrations this tended to switch to the "natural" base line. So in order to produce proportional, relatively linear standard curves two integrator settings would have been required and two sets of standard curves; one for high concentrations and one for low concentrations. It was considered best to avoid the use of two standard curves for each compound otherwise this would have lead to the preparation of eight standard curves. The net result was that standard curves showed a curvature and that peak height was more robust to the change in reference baseline than peak area; ie. the peak height standard curve showed the least curvature. Hence, peak height measurement was the method of choice.

7.7.2 Batch Reaction Conditions and Buffer Choice

Reaction conditions were selected on the basis of results obtained in earlier experiments and on published data, especially that of Lemiere et.al. (1985). The conditions selected were: organic phase- 10 mM octan-1-ol in 100 ml, 2,2,4-trimethyl pentane and; aqueous phase- 0.001 M NAD^+, 0.1 M acetaldehyde in 100 ml, 0.1 M

pH 9.5 NaOH-glycine buffer.

3 membrane cassettes (area 180 cm^2) were placed in the reactor and loaded in the manner described in Section 7.6. The aqueous and organic phases were added to their appropriate reservoirs, their flow rates set to 6.0 ml/min and 4.0 ml/min respectively and the run started. The experiment was carried out at room temperature. Within a few hours of beginning the run the aqueous phase had become a clear, deep straw brown in colour. A sample of the aqueous phase that had not been used in the experiment was also the same colour. The experiment was abandoned and the phenomenon investigated.

Following advice from Dr. T. Gallagher (1990) an interaction between the buffer agent (glycine) and acetaldehyde was suspected and in fact a solution of 0.1 M acetaldehyde in 0.1 M, pH 9.5 buffer produced an identical colour change to that previously observed. Hence, a new buffer was needed and a sodium carbonate-sodium hydrogen carbonate system was chosen. A new solution of the aqueous phase was prepared with this buffer and left overnight. After 24 hours only a light straw coloration had developed suggesting that the reaction of acetaldehyde with other constituents of the aqueous phase had been minimised.

7.7.3 Batch Experiments with Coenzyme Regeneration

5 membrane cassettes (area 300 cm^2) were used. The enzyme was loaded and

the membranes washed as described in Section 7.6. The reaction conditions selected were: organic phase- 5 mM octan-1-ol in 100 ml 2,2,4-trimethyl pentane and; aqueous phase- 0.001 M NAD^+, 0.1 M acetaldehyde in 0.1 M, pH 9.5 Na_2CO_3/HCO_3 buffer. The reaction was carried out at room temperature which was measured. The phases were loaded into their reservoirs, the aqueous and organic phase flow rates were set to 6.0 ml/min and 4.0 ml/min respectively and the reaction begun. The reaction was allowed to proceed for 24 hours with monitoring of the aqueous phase absorbance at 340 nm and regular sampling of the organic phase for GLC analysis.

After the membranes had been regenerated and were fit for use the same experiment was repeated except that an excess amount (2 mg) of yeast alcohol dehydrogenase (YADH) was added to the aqueous phase at the start of the experiment. This was added to aid the regeneration of NAD^+.

Graphs of comparable experiments for each of these systems are displayed as Figures 7.13 and 7.14. Both systems displayed comparable productivity of octyl aldehyde and similar concentrations of acetaldehyde in the organic phase but, differed substantially with respect the concentration of ethanol in the organic phase which was approximately 4 fold lower in the system containing YADH than in the corresponding system containing no YADH.

7.7.4 Repetitive Batch Experiments

A series of runs were undertaken which used the same aqueous phase to assess the longevity and productivity of coenzyme regeneration. In the previous two coenzyme regeneration experiments the ratio of the starting concentration of octan-1-ol to NAD^+ was 5:1 and so even if the conversion of octan-1-ol to octyl aldehyde were 100% then the total turnover number for NAD^+ (moles product/moles NAD^+) could not have exceeded 5. So, by performing repetitive batch experiments using the same aqueous phase it was hoped to markedly increase the total turnover number for NAD^+.

The enzyme was immobilised as described in Section 7.6 and the reaction conditions were the same as those described in Section 7.7.3 except that 2.7 mg of YADH was included in the aqueous phase. This was incorporated into the aqueous phase as an attempt to ensure that the concentration of the regenerating agent, acetaldehyde, remained high over the course of the runs to provide a continual driving force for the regeneration reaction.

After 24 hours the pumps were turned off and a final sample of the organic phase taken for GLC analysis. The rig was then drained of organic phase by reversing the organic phase pump. During this process a proportion of the aqueous phase was drawn into the organic phase reservoir. This was simply withdrawn using a pipette and placed back in the aqueous phase reservoir. The volume of the recovered organic phase was measured before it was discarded and the reservoir was rinsed twice with

2,2,4-trimethyl pentane.

The GLC analysis revealed the final concentration of acetaldehyde present in the organic phase to be 4mM. Because the experiment involved the multiple replacement of the organic phase this meant that the acetaldehyde concentration of the reactor would be depleted steadily with each run. Therefore, to solve this problem and to keep the acetaldehyde concentration at approximately its original level each fresh 100 ml organic phase was made 4 mM in acetaldehyde.

A fresh, 100 ml of organic phase containing 5mM octan-1-ol and 4mM acetaldehyde in 2,2,4-trimethyl pentane was added to the reactor and the reaction allowed to run again for a further 24 hours. In all, 4 runs were carried out in this manner.

For the fifth run a new aqueous phase was also added containing 2 mg YADH. This was added to establish whether reduction of the reaction yield (which was observed over the first four runs) was due to loss of stability of the enzyme or a change in the nature of the aqueous phase due to its repetitive use (e.g. NAD^+ is prone to decomposition in alkaline solution Chenault and Whitesides 1987). A rejuvenation of the reaction rate on addition of the new aqueous phase might have been expected if the latter was occurring.

From these experiments a total yield of 1.356 mMoles of octyl aldehyde was obtained over the course of the five runs. The turnover number of NAD^+ (moles

product/moles of NAD^+ used, for the first 4 runs only) was 11.72.

Stability of the enzyme over the total course of the experiment and change in the aqueous phase absorbance at 340 nm for each run are plotted as Figure 7.15. The time course profiles of each of the runs are included for reference as Appendix 3. From Figure 7.15 it can be seen that the operational stability of the enzyme was higher than that observed previously for the membrane immobilised enzyme ($t_{1/2}$= +120 hours). As the reaction yield was not increased by the addition of a fresh aqueous phase on the fifth run this supports the idea that the reduction of product yield over time was indeed due to an actual reduction of enzyme stability. Increased stability was probably due to operational stability and this was considered to have brought about by increased, uniform enzyme loading as discussed previously in Section 7.6. This would have enhanced mass transfer limitations as mentioned and therefore caused the enzyme to function substantially below full capacity. As the enzyme stability reduced this would not have affected the reaction rate until the active enzyme concentration was below the reaction capacity dictated by the substrate availability. Also there was the possibility of continuous catalytic activity of the enzyme conferring increased stabilisation of the enzyme structure. This effect was reported for HLADH by Villame et.al.(1990).

7.8 SUMMARY

The Minitan® unit was demonstrated to operate as a two-phase reactor

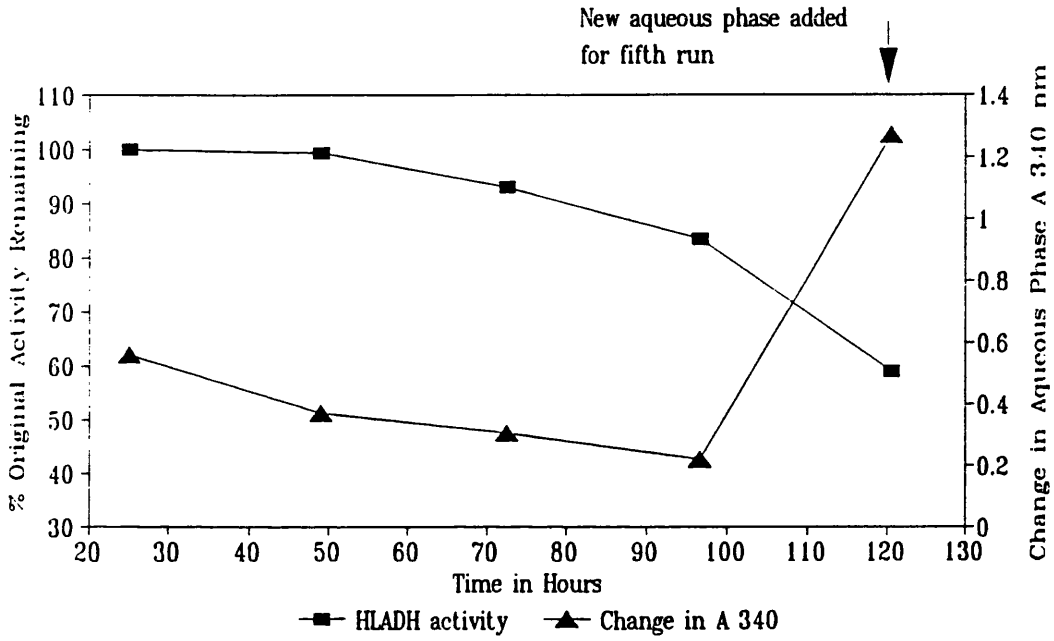


Fig 7.15. A Plot Showing the Immobilised Enzyme Stability and Change in A 340 nm During the Repetitive Batch Runs using the Minitan Unit.. HLADH activity was obtained by dividing the final yield of octyl aldehyde by the time period of reaction to give a reaction rate as µMoles octyl aldehyde/ minute.

allowing both production of octyl aldehyde (as confirmed by GLC) and coenzyme regeneration. The system was developed into a test reactor and analysis techniques were developed to monitor the progress of reactions.

Investigation of two coenzyme regeneration strategies revealed that a regeneration support enzyme (YADH) did not increase the rate of octyl aldehyde formation.

Repetitive batch experiments demonstrated an increased level of enzyme stability due to operational stability effects and possibly to an enhancement of stability by continuous catalysis.

CHAPTER 8

DISCUSSION

8.1 INTRODUCTION

The reactor developed in this thesis was an extension of work by Hoq et.al. (1985) who used an aqueous, organic solvent, two-phase membrane reactor to hydrolyse and synthesize lipids using lipases. In Hoq's reactor a membrane was used as the enzyme support and as means to keep the two phases discreet. This type of reactor proved commercially significant and has been tested at pilot scale as a means of synthesising cocoa butter from palm oil.

These reactors have used only simple "hydrolase" enzymes as the biocatalyst but by incorporation of a coenzyme into the aqueous phase the range of useable enzyme systems could be extended to include oxidoreductases. The feasibility of such a reactor was considered worthy of investigation as oxidoreductase enzymes are capable of regio- and stereo-specific transformations of commercially important classes of aqueous insoluble (hydrophobic) compounds eg. steroids.

Further, as oxidoreductase enzymes require stoichiometric quantities of high cost coenzyme (NAD^+ or NADH) regeneration of the coenzyme was considered to be an essential component of a reactor. It has been shown (Chenault and Whitesides 1987) that with the use of a regeneration system coenzyme cost can be reduced to a

small fraction of total process costs.

So, the design of the reactor consisted of: an appropriate enzyme; an aqueous phase containing a coenzyme and its regeneration system; an organic phase containing hydrophobic substrate; and a membrane separating the two phases and acting as a support for the immobilised enzyme.

This project represented a feasibility study to examine the potential of this type of reactor. The approach taken can be broken down into several parts:

1) Selection of a model reaction and demonstration that the reaction proceeded at a reasonable rate;
2) Analysis of the factors important in controlling reaction rate;
3) Performing the reaction in a test reactor and incorporation of coenzyme regeneration.

Choice of the model reaction system was discussed in Section 1.8.2 and the work up to the demonstration of this reaction is outlined in Chapter 3. Experiments to characterise factors controlling the reaction rate were carried out in Chapters 4, 5 and 6. The implications of results obtained from these experiments are now considered in further detail.

8.2 CHARACTERISATION OF THE REACTION SYSTEM IN THE SMALL GLASS REACTOR AND FACTORS AFFECTING THE REACTION RATE

8.2.1 Examination of the Kinetics of Soluble HLADH using the Michaelis-Menten Equation

These experiments were conducted as a control so that modification of the enzyme's kinetics by immobilisation were easily observed.

Most experiments were carried out at pH 9.5 as this was the optimum pH identified (see Figure 4.9). The results obtained showed conclusively that initial reaction enzyme kinetics of soluble enzyme followed closely Michaelis-Menten kinetics for an irreversible, two substrate reaction. This was demonstrated by a close match between experimental and model results plotted as v against S and v/S against v (Figures 4.4 to 4.5 and Figures 4.7 to 4.8 respectively). Values of kinetic constants for the soluble enzyme were similar to literature values (Sund and Theorell 1963, Martinek et.al. 1982).

In short the results obtained for the soluble enzyme were clear cut and agreed with previous findings. These results provided a firm basis to which behaviour of the immobilised enzyme could be compared.

8.2.2 Examination of the Kinetics of Immobilised HLADH using the Michaelis-Menten Equation

The immobilised enzyme was first examined using the same Michaelis-Menten analysis applied to the soluble enzyme. When experimental results were examined alongside model results as plots of v against S it became clear that the Michaelis-Menten equation was an inaccurate model of the immobilised enzyme's kinetic behaviour (see Figures 5.1 and 5.2).

It was possible that deviations from the Michaelis-Menten case were the result of experimental variation. Experiments with the immobilised enzyme were more complex than those for soluble enzyme and were prone to a greater range of experimental error. Sources of error beyond those experiments with the soluble enzyme were subject to included errors from: the protein assay; daily temperature changes; the use of different batches of NAD^+ solutions each day; and possible changes in the membrane from its repeated regeneration.

All of these were sources of random error and so would have produced a random deviations of results from predicted Michaelis-Menten behaviour. However, for each octan-1-ol concentration results showed a relatively orderly change in reaction rate with NAD^+ concentration (see Figures 5.1 and 5.2). From this it was suspected that deviations from the Michaelis-Menten case were more likely to have been the result of a kinetic mechanism.

To test this idea further Eadie-Hofstee plots (ie. plots of v/S against v) were used as a diagnostic tool. If results were plagued by random error then plots would have shown a high degree of scatter. If, on the other hand deviations were a kinetic phenomenon then the plots would show systematic deviations from linearity.

Results from this analysis (Figure 5.3 and 5.4) clearly showed systematic deviations from linearity and this strongly suggested a kinetic mechanism was responsible for deviation from the simple Michaelis-Menten case. Given the absence of significant deviations for the soluble enzyme then the observed deviations must have arisen as a consequence of immobilisation and reaction within the confines of the membrane support. This conclusion suggested further experiments to identify the mechanisms responsible for the observed kinetics. The approach used was a systematic examination of the response of immobilised enzyme kinetics to physicochemical changes of reaction conditions.

8.2.3 Evidence for the Contribution of Mass Transfer Resistance to the Kinetic Behaviour of the Immobilised Enzyme

The most unequivocal indication of mass transfer limitation of the reaction was given by the effect of varying membrane pore size (see Figure 6.1 and Section 6.2) The specific reaction rate with the 0.2μm membrane was approximately 60% of that with the 0.65μm membrane.

This result was obtained under conditions where external mass transfer limitations had been shown to be minimised by use of suitable stirrer speeds (see Section 3.4.6) so the mass transfer effects observed must have been due to internal mass transfer limitations. As there were a total of 5 substrate/products for the reaction this made the system relatively complex so it was not possible to glean from this result which transfer rates were contributory to the mass transfer effect.

The protein recovery results from experiments in the small, glass reactor (Table 3.1) also showed some evidence of internal mass transfer limitation (again the experiments were conducted under conditions where external mass transfer had been minimised). The results showed that as the amount of protein recovered varied the reaction rates remained relatively constant. This suggested that substrate/product transfer rates influenced the reaction rate because these would have remained constant from experiment to experiment whilst the concentration of the enzyme varied.

Although the Eadie-Hofstee plots (Figures 5.3 and 5.4) showed that an increase in the substrate concentrations reduced the observed deviation from Michaelis-Menten kinetics this relationship cannot be taken as evidence of internal mass transfer limitation of the reaction per se. This was because other factors such as partitioning of substrate from the organic phase into the membrane also probably contributed substantially to this effect.

8.2.4 Diagnosis of the Immobilised Enzyme Kinetics by Examination of pH Effects

The effect of pH on reaction rate showed the pH profile of the immobilised enzyme to be disturbed compared to that of the soluble enzyme (see Figure 5.9). The evidence strongly indicated a shift in the local pH of the enzyme to relatively acidic conditions. The net charges of substrates and products were: octan-1-ol and octyl aldehyde uncharged; NAD^+(-1); NADH(-2); and H^+ ions(+1). Obviously, of these compounds only H^+ ions could have reduced the local enzyme pH.

Given the prevailing mass transfer limitation outlined above it was probable that mass transfer limitation of H^+ ion escape was a mechanism which contributed significantly to reduction of the local pH of the enzyme. As the intramembrane buffer was likely to have unstirred transport of H^+ ions from the membrane would have depended on diffusion ie. controlled by the internal mass transfer rate. On initiation of reaction it was probable that H^+ ions rapidly accumulated in the tiny internal membrane volume ($<$ than 100μl) and reduced the pH of buffer within the membrane to a relatively acidic value. This would have changed the pH of the enzyme's microenvironment and reduced the reaction rate to the level observed.

A similar pH effect was described by Roig et.al. (1990) who also used HLADH immobilised on PVDF membranes but, the work was carried out using a wholly aqueous system with ethanol as the alcohol substrate. A similar explanation of the result was provided by Roig et.al.

It was also likely that membrane charge was contributory to the pH effect. It was found that the membrane neutralised NaOH at pH 9.5 (see Figure 6.7) indicating it was positively charged. So it was likely that immobilisation of the enzyme was partly electrostatic and that the enzyme was per se in a relatively more acidic environment than that of the pH 9.5 buffer.

Further, it was likely that a positive membrane charge moderated internal mass transfer limitation of the rate of H^+ ion escape. Like charges of the membrane and H^+ ions would have produced a partition driving force acting to expel H^+ ions from the membrane. This would have lead to H^+ ion expulsion rates greater than those capable of being produced by the H^+ ion internal mass transfer rate alone.

8.2.5 Diagnosis of the Kinetics of the Immobilised Enzyme by Examination of Ionic Strength Effects

There were a total of 5 substrates and products associated with the reaction of which 3 possessed net charges (NAD^+(-1), NADH(-2) and H^+ ions(+1)). As high ionic strength buffers tend to saturate and suppress charge effects then by increasing ionic strength it was possible to examine the net charge effects of these compounds and the membrane charge on the kinetics of the immobilised enzyme.

The results (Figure 5.8) clearly showed increasing ionic strength of the aqueous phase buffer decreased reaction rate whilst the soluble enzyme showed no

appreciable change in reaction rate.

In low ionic strength buffer charge partition effects were likely to have been present where compounds of like charge to the membrane (H^+ ions) would tend to have been repelled from the membrane and compounds of opposite charge (NAD^+ and NADH) would tend to have been attracted to the membrane.

At high ionic strength the charges of these compounds and the positive charge of the membrane would have been significantly reduced. In terms of the reaction, suppression of charge partitioning would have: 1) reduced the tendency for NAD^+ to partition favourably into the membrane; 2) reduced the tendency for NADH to partition unfavourably into the membrane; and 3) reduced the concentration of H^+ ions within the membrane. Obviously if the rate of transfer of NAD^+ was reduced then the reaction rate would have been lowered as was observed.

Further to these charge partition effects the transfer of NAD^+ and NADH in and out of the membrane will probably have been influenced considerably by internal mass transfer limitations. This is a reasonable conclusion given the evidence for mass transfer limitation of H^+ ion escape discussed above because both NAD^+ and NADH were of much higher molecular weight and thus would have possessed much lower diffusion rates than H^+ ions. So the membrane probably functioned to influence the reaction rate at two levels. These were: mass transfer limitation of the transport rate of probably all substrates and products within the membrane; and superimposed on this was partitioning of probably all substrates and products between the membrane

and the corresponding bulk phase.

With respect to the aqueous phase substrates and products, reduction of charge effects at high ionic strength would have allowed their intramembrane concentrations to be determined mainly by internal mass transfer rates. The result was a decrease of the overall reaction rate which suggested the influence of membrane charge was to offset the severity of internal mass transfer limitation of the reaction rate. Given this explanation of the mechanism responsible for the ionic strength effect then retention of a positive charge on the membrane was beneficial to the reaction rate.

8.2.6 Estimation of the Relative Importance of Internal Mass Transfer and Partition Effects

Overall the effect of ionic strength probably demonstrated a shift between the relative importance of partition effects and internal mass transfer limitations. By examining the maximum change in reaction rate attributed to the presence and reduction of these two effects it was possible to approximate their relative importance to control of reaction rate. Although this analysis was not considered to have been conducted against thorough control experiments (eg. the effect of membrane pore size was not conducted at high ionic strength where partition effects would have been minimised) it did allow a semiquantitative comparison of mass transfer and partition effects.

From the results for the investigation of pore size the reaction rate obtained using a 0.22μm membrane was 0.023 μMoles/min/mg HLADH. The reaction rate obtained under experimental conditions where internal mass transfer limitations were considered to have been minimised (the 0.65 μm membrane) was 0.037 μMoles/min/mg HLADH. So by taking a ratio of the two then the effectiveness of the reaction with the 0.2μm membrane was 0.62 (ie. internal mass transfer reduced the reaction rate by 38%). Further, because the reaction rate was still increasing with pore size at the maximum pore size used (0.65μm) it was very likely that internal mass transfer limitations were still significant at this pore size and so, the reduction of reaction effectiveness was probably underestimated.

From the results for the effect of ionic strength the reaction rate obtained under conditions where partition effects were considered to have been the most pronounced (0.1 M buffer) was 0.022 μMoles/min/mg HLADH. The reaction rate where this effect was minimised (1.0 M buffer) was 0.016 μMoles/min/mg HLADH. So under typical reaction conditions (ie. 0.1 M buffer) the effectiveness of the reaction in the presence of partition effects was 1.375 (ie the aqueous phase partition effect increased the reaction rate by 38%). This result is expressed as a value greater than unity because the partition effect was considered to have a positive effect on the reaction rate.

From comparison of these results it appeared that the partition and mass transfer effects were of similar magnitude. However, it was probable that transfer within the membrane was still a diffusion process even in the 0.65μm membrane and

so the effectiveness value for internal mass transfer was probably optimistic. This conclusion suggested internal mass transfer was the most significant influence on the overall reaction rate.

With respect to transfer of the organic phase substrate and product, little quantitative information was obtained. However, partitioning of these compounds would have been present per se as these were hydrophobic compounds which had to dissolve in and pass through an aqueous layer to approach active enzyme and vice versa. So partition effect were probably severe. However, the transfer rate obtained from experiment which measured the transfer rate of octan-1-ol across the membrane into the bulk organic phase (Section 6.5) showed that the transfer rate occurred approximately 10 times faster than the reaction rate. This gave some indication that at this concentration (1% octan-1-ol) the octan-1-ol transfer rate was probably not the major rate limiting step. This situation only served to highlight the relatively low reaction rate of the immobilised enzyme. This conclusion is only tentative and the experiment was considered worthy of inclusion only as it served to indicate how organic phase transfer rates might be investigated further in future work.

The Eadie-Hofstee plots (Figures 5.3 and 5.4) supported the conclusion in the above paragraph regarding the effect of octan-1-ol concentration on reaction rates. The plots showed that deviations from Michaelis-Menten kinetics were reduced with increase in the bulk organic phase concentration. At the octan-1-ol concentration used in the mass transfer experiment (1% or 63000 μm) curvature of the plot was much reduced. With respect to octan-1-ol, curvature of the plots was probably produced by

a combination of partition and mass transfer effects. From the results obtained these two effects could not be separated and so their relative contributions to the availability of octan-1-ol were not measured.

8.2.7 The Magnitude of Reaction Rate of the Immobilised Enzyme and Enzyme Stability

The reaction rates demonstrated by the immobilised enzyme were generally 100 fold lower than those of the soluble enzyme. Further, although the partition and mass transfer effects discussed above did modulate reaction rates none of the reaction rates observed approached that of the soluble enzyme. This strongly suggested the bulk of the immobilised enzyme was inactivated during the immobilisation process. This was probably a direct result of the character of the enzyme attachment and given the fact that the membrane was charged it is tempting to speculate that the immobilisation processes was at least partially electrostatic and that this interaction may have disrupted the enzyme structure leading to its inactivity. However, beyond the fact that the activity reduction occurred on immobilisation no evidence was obtained to confirm this idea.

A comparison of the immobilised and soluble enzyme stability was given in Table 5.1 where enzyme half lives were compared. The results showed that the immobilised enzyme was less stable than its soluble counterpart in pH 8.0 and 9.5 buffers, also, the pH conditions for optimal stability differed. The observation of

optimal stability of the immobilised enzyme at pH 9.5 (cf. 8.0 for the soluble enzyme) gave further suggestion that the enzyme was operating in an acidic environmemt relative to the soluble enzyme ie. the pH stability profile of the enzyme was artificially shifted toward a higher pH because the membrane interior was maintained at a lower pH than the bulk solution by a combination of membrane charge and mass transfer effects.

The shape of the activity decay curves, for soluble and immobilised enzyme (Figures 4.11 and 5.12 respectively) in most cases suggested simple, 1st order, exponential decay. Therefore, it seemed possible that immobilisation simply accelerated the denaturation mechanisms responsible for loss of soluble enzyme activity. Again it might be speculated that membrane charge was responsible for the comparative destabilisation of the immobilised enzyme. Retrospectively, the rates of decrease of activity observed and the relative inactivity of the immobilised enzyme were not particularly surprising as Villaume (1990) states that one of the recognised problems in investigations of HLADH activity is its poor stability.

It must be recognised that although the reaction rates obtained were too low to be of commercial interest they were sufficient to allow examination of the feasibility of the overall system. The fact that a measurable reaction occurred and the phases remained discrete allowed identification of factors which would be important in the development of a viable reaction system. These results provided a framework for a programme to evaluate the potential of a larger scale reactor including coenzyme regeneration.

8.3 EXPERIMENTS IN THE MINITAN® REACTOR

8.3.1 Introduction to Experiments in the Minitan® Reactor

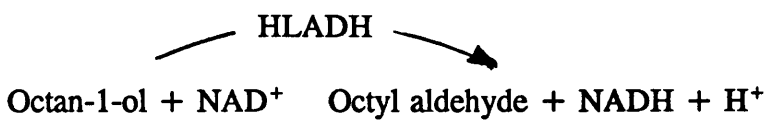
The small glass reactor was designed to allow reaction rates to be followed by monitoring changes in the aqueous phase absorbance resulting from the reduction of NAD^+. For this reason it was unsuitable for following reactions incorporating coenzyme regeneration where changes in the aqueous phase absorbance were not directly related to the stoichiometric reaction. A reactor based on a Millipore Minitan® microfiltration rig was designed to overcome these problems. The development of the reactor and the supporting analytical methods are presented in Chapter 7.

8.3.2 Batch Experiments with Coenzyme Regeneration

Two coenzyme regeneration strategies were examined in batch experiments. The systems were compared for: rate of octyl aldehyde formation; the reaction stoichiometry; and the fractional conversion of octan-1-ol.

The first system used the immobilised HLADH as the reaction and coenzyme regeneration catalyst. To do this it was only necessary to add the regeneration system substrate- acetaldehyde. The full reaction system is:

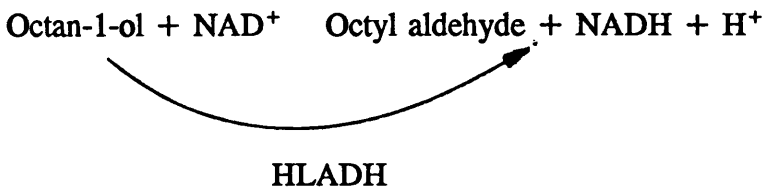
Product reaction;



Regeneration reaction;

Batch reaction, time course profiles for the systems utilising HLADH and YADH as the regenerating agents are presented as Figures 7.13 and 7.14 respectively. The most important feature of these profiles are that both showed curved product accumulation traces with yields of approximately 90% octyl aldehyde (octyl aldehyde was confirmed as the product by GLC). From plateauxing of octyl aldehyde accumulation in these graphs it was observed that in both cases the reaction was close to its equilibrium. The concentrations of octyl aldehyde shown in the graphs was the total yield as octyl aldehyde was relatively insoluble in buffer and so was not detected in the aqueous phase by GLC analysis. For this reason the concentration of octyl aldehyde was expressed in terms of the organic phase concentration. For consistency the concentrations of octan-1-ol, ethanol and acetaldehyde were also expressed with reference to the organic phase volume. In retrospect the decision to measure octyl aldehyde and ethanol in the organic phase probably obfuscated the results because these compounds partitioned less selectively between the phases than octyl aldehyde and lead to measurement of only a portion of their overall concentrations. For example, the concentration of acetaldehyde measured in the organic phase was

Product reaction;



Regeneration reaction;

Although this regeneration strategy has the advantage of being very simple it does possess an inherent problem in that both the regeneration reaction and the product reaction were to be catalysed simultaneously by the same enzyme. Thus the potential capacity of the system to produce octyl aldehyde was reduced as acetaldehyde and ethanol produced by regeneration would have competed with octan-1-ol for utilisation of the enzyme.

The second system examined was an attempt to examine this inherent problem. In the second regeneration system yeast alcohol dehydrogenase (YADH) was added to the aqueous phase along with acetaldehyde and was used to catalyse coenzyme regeneration. YADH is specific for the conversion of acetaldehyde to ethanol, so by including it in the aqueous phase it was hoped it would perform some degree of uncoupling of HLADH from the regeneration system and lead to higher rates of octyl aldehyde production. The full reaction is:

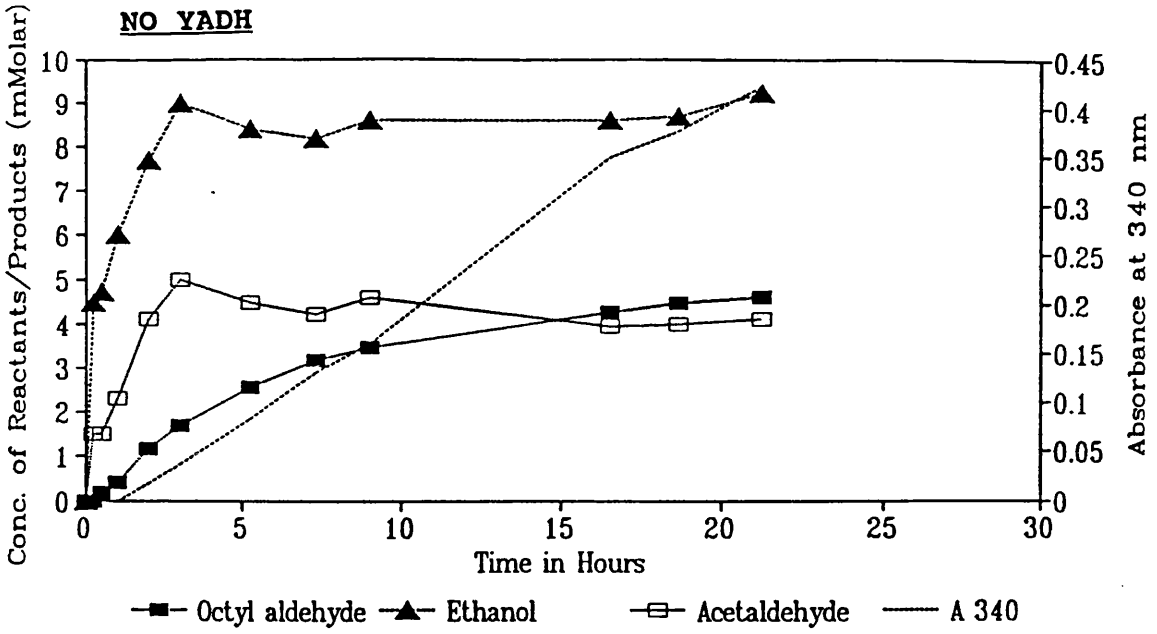


Fig 7.13. Time Course Profile for a Regeneration Experiment in the Mintan Rig. Conditions were: 5, 0.2µm plates (18 mg total HLADH recovered); aqueous phase– 100 ml 0.1 M sodium carbonate / bicarbonate buffer containing NAD @ 1mM and acetaldehyde @ 0.1 M; organic phase– 100 ml, 2, 2, 4– trimethyl pentane containing octan–1–ol @ 5 mM; temperature– 18°C. All concentrations were measured in the organic phase except A 340 nm.

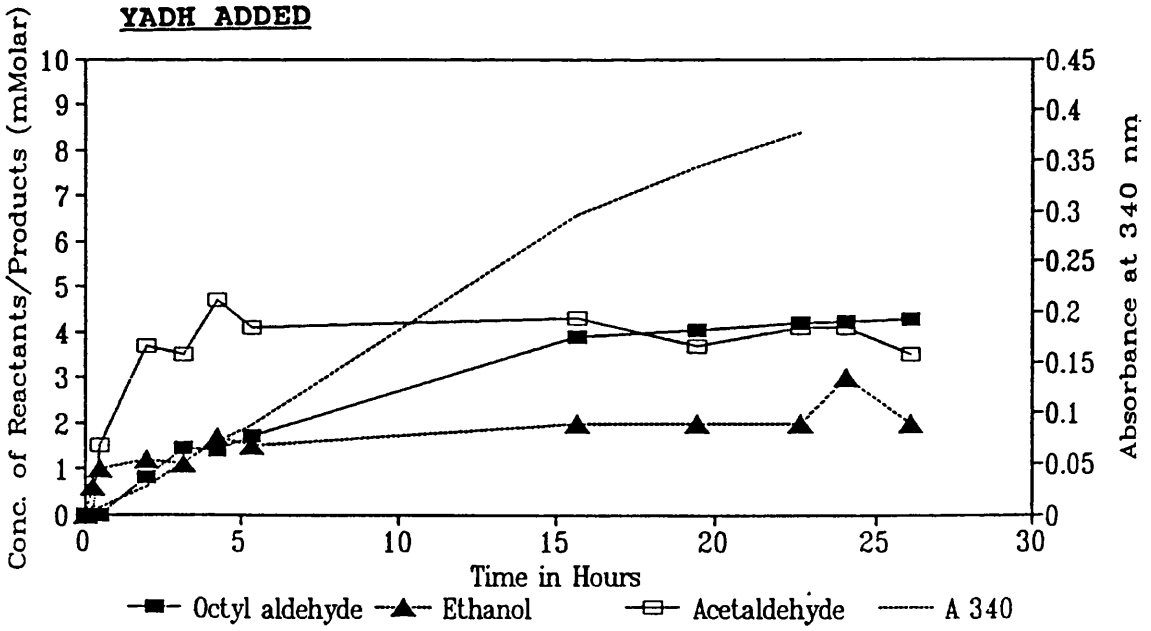


Fig 7.14. Time Course Profile for a Regeneration Experiment in the Minitan Rig. Conditions were 5, 0.22µm plates (10.67 mg total HLADH recovered); aqueous phase– 100 ml 0.1 M sodium carbonate / bicarbonate buffer containing NAD @ 1mM, acetaldehyde @ 0.1 M and 2 mg yeast ADH; organic phase– 100 ml 2, 2, 4 trimethyl pentane containing octan–1–ol @ 5 mM; temperature 18°C.. Concentrations were measured in the organic phase except A 340 nm.

approximately 4 mM when its initial concentration in the aqueous phase was 0.1 M.

In both reactions octyl aldehyde appeared at approximately the same rate and reached approximately the same concentration. This demonstrated that the incorporation of YADH was ineffectual ie. its inclusion did not change the rate of the reaction. On examination of this result in the light of the evidence for mass transfer limitation of the reaction and partition effects then perhaps this was not surprising. These rate controlling mechanisms would have functioned to limit the potential of regeneration by YADH, as YADH was not present in the membrane then it could only regenerate NADH liberated by diffusion against the ionic charge driving force (see Section 8.2.5) into the bulk aqueous phase and so in this sense it could not have functioned to circumvent these reaction rate limiting mechanisms.

The reaction course profiles also showed the accumulation and partitioning of the regeneration product ethanol into the organic phase and the partitioning of acetaldehyde between the phases. In both experiments these compounds reached stable concentrations in the organic phase within 5 hours indicating that a partition equilibrium was being maintained in each case.

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The reaction course profiles also showed accumulation and partitioning of the regeneration product ethanol into the aqueous phase and partitioning of acetaldehyde between the phases. In both experiments these compounds reached stable concentrations within five hours indicating that a partition equilibrium was being maintained in each case. Further the graphs also showed the organic phase concentration of ethanol (and consequently the overall concentration of ethanol for the system) was lower in the run incorporating YADH (cf. ethanol concentrations in Figures 7.14 and 7.15). This suggested it was possible that instead of regenerating NAD^+, YADH actually utilised some NAD^+ and produced acetaldehyde.

In the system containing HLADH as the regenerating enzyme, initially

reaction would have produced NADH and octyl aldehyde. The octyl aldehyde would have selectively partitioned into the organic phase and thus would not have been available for the back reaction. NADH would have reacted with acetaldehyde and produced ethanol within the membrane. On continuation the reaction settled down to a steady state, recycling the coenzyme between NAD^+ and NADH utilising mainly acetaldehyde and ethanol. The changing concentrations of octan-1-ol and octyl aldehyde would have had little effect on this steady state being present at a maximum of 5 mM against a steady state established with 0.1 M acetaldehyde. Toward the end of the reaction, the octyl aldehyde concentration built up in the organic phase and so it became increasingly available within the membrane for recycling of coenzyme, ie. the reaction approached an overall equilibrium (and the reaction rate slowed), with the equilibrium determined by the aqueous phase concentrations (NAD^+ and NADH are insoluble in hydrophobic organic solvent and so the organic phase concentrations had no bearing on the equilibrium position).

The inclusion of YADH in the second run was an attempt to uncouple HLADH from regeneration of coenzyme and so increase the reaction rate for the production of octyl aldehyde. This did not occur for the reasons outlined above but, it inclusion did appear to alter the equilibrium concentration of ethanol with respect to the experiment utilising HLADH as the regenerating enzyme. This difference was not clearly understood (and the experiment was not conducted in replicate so the result was not conclusive) but, it was considered possible that it was an effect of the membrane where partitioning and mass transfer limitations would have lead to a difference in operating environments between the enzyme systems. It was possible

that at pH 9.5 ethanol formation by YADH was so unfavourable that, in spite of the initial presence of 0.1 M acetaldehyde, ethanol produced by HLADH within the membrane was converted to acetaldehyde on liberation into the aqueous phase and so its concentration remained low. Ethanol production within the membrane by HLADH regeneration system may have been more favourable because of the lower pH of the intramembrane buffer and so more ethanol would have been liberated into aqueous phase where it could not have been converted to acetaldehyde . In It is known that both HLADH and YADH switch from aldehyde production to alcohol production as pH is reduced from approximately 9.5 to 7.0 (Brändén et.al. 1975, Sund and Theorell 1963).

Fractional conversions of octan-1-ol were approximately 0.9 in both systems. This gave total turnover numbers (TTN) ie. product in moles / starting NAD^+ in moles, of approximately 4.5 which indicated that coenzyme recycling occurred in both reactions (the maximum theoretical TTN was 5 as NAD^+ and octan-1-ol were included at 1 and 5 mM respectively). The fractional conversions obtained were possibly improved over reaction in aqueous system, although this was not tested. In a single phase system products accumulate in the same environment as the reactants and the equilibrium position of the reaction can reflect this and give fractional conversions much less than 1. In this reaction the octyl aldehyde was sparingly soluble in the aqueous phase and very soluble in the organic phase. This contrasted with the solubility of NADH which being a polar molecule was practical insoluble in the organic phase. Thus these two reaction products were physically separated with the organic phase acting as a sink for octyl aldehyde produced by reaction and so in

comparison to a single phase aqueous system, the equilibrium was probably tipped in favour of octyl aldehyde and NADH.

Due to the selective partitioning of products, it can be suggested that a two-phase reaction scheme using an organic-insoluble coenzyme has the potential to yield close to 100% product from a bioconversion. Further, as the enzyme is usually in the aqueous phase and this phase is the only place where all the reactants and products come into proximity then the equilibrium position is determined by aqueous phase concentrations. Hence, the factor deciding the final equilibrium position would be the solubility of the organic phase product (ie. the greater its solubility in the organic phase then the further in favour of products the equilibrium position would be moved. Possibly the type of reaction best suited for two phase biocatalysis would be the conversion of an aqueous soluble product into an aqueous-insoluble product (eg. the conversion of fatty acids and glycerol to lipids by Hoq et.al. 1985).

8.3.3 Repetitive Batch Experiments in the Minitan® Reactor

From the previous results it was evident that the reaction rate could not have easily supported the implementation of a continuous reaction regime as the dilution rate required would of been too low to allow simple and accurate control under practical conditions. Hence repetitive batch reactions were performed as an alternative approach for investigating the stability of the system.

In these reactions acetaldehyde was added in the organic phase at the start of reactions 2-5. This was required to replace the acetaldehyde removed from the reactor with the previous organic phase. The quantity added was simply the equilibrium concentration of acetaldehyde that had been observed in the previous two batch experiments (approximately 4 mM). Also, the experiments were run with the inclusion of YADH to prevent a continual increase in the concentration of ethanol and to allow a maintained acetaldehyde concentration. However, in the light of the discussion above, this was probably not justified.

Appendix 3 shows the reaction progress curves for the series of experiments. From these it can be seen that the addition of acetaldehyde in the second run onwards initially produced a high organic phase concentration but this rapidly stabilised to a value representing its partition equilibrium. The ethanol concentration behaved in a similar manner increasing rapidly and then stabilising at its partition equilibrium. The stability of the concentrations of these two compounds also suggested an equilibrium between NAD^+ and NADH.

The Figures also show the increase in the organic phase octyl aldehyde concentration and aqueous phase absorbance (N.B. at the start of each run the aqueous phase absorbance was rezeroed). These measurements showed a relationship to each other over the time course of each of the first four runs. The change in aqueous phase absorbance also parallelled the decrease in octyl aldehyde production over the first four runs. This was surprising given that the aqueous phase absorbance was thought to be determined by the ratio of NAD^+ to NADH present and possibly

the absorbance of products of side reactions. It was not clear how either of these factors could have been proportionately linked to octyl aldehyde production and reason for the relationship were not uncovered.

The results presented as Figure 7.15 showed the stability profile of the enzyme. Compared to the results obtained earlier for the stability of the immobilised enzyme (Figure 5.12) then it is apparent that the enzyme was at least 4 times more stable in the repetitive batch reactions. There were several contributory factors which might account for this.

Firstly, there was a slight carry over of the organic phase between the batch reactions (< 10% volume), hence this factor was responsible for a slight distortion of the stability profile.

Secondly, the enzyme was continuously catalytically active in these experiments whereas in the earlier experiment the enzyme actively was merely tested every day. Therefore, due to the influence of the reaction on the membrane pH, the enzyme was effectively at a pH value lower than the bulk solution and in this way continuous reaction apparently stabilised the enzyme. This implies that the stability profile was more aptly comparable to the profile obtained at pH 8.0 in Figure 5.12. However, this comparison revealed that the stability of the enzyme in the repetitive batch reaction was still unmatched.

In fact, there was probably a stabilisation of the enzyme due to its continuous

catalytic activity alone. Recently, Villaume et.al. (1990) presented results showing that this was the case for HLADH. These workers showed increased stability of HLADH by continuous catalytic activity and suggested the increase in stability occurred because the enzyme changed conformation during catalysis and the active state was the more stable conformation.

Finally the reaction probably demonstrated a degree of operational stability. This is the situation where, because of mass transfer limitations, not all of the enzyme is catalytically available at the onset of the reaction and despite inactivation over the course of the reaction there is still enough active enzyme present to match the mass transfer rate. This situation makes an enzyme appear more stable than it actually is. Although this phenomenon was not seen in the stability experiment in the small, glass reactor (the rate dropped continually at each successive measurement) it may well have occurred in the Minitan® reactor.

As explained in Section 7.6 there was probably a difference in the location of the enzyme between experiments in the small, glass reactor and the Minitan® reactor whereby in the Minitan® reactor the enzyme was more evenly distributed throughout the membrane (more enzyme being present within the centre of the membrane). This made the membranes used in the Minitan® reactor more susceptible to mass transfer limitations (for the further discussion of this see Sections 6.2 and 7.6). This in turn opened up the possibility for operational stabilisation of the enzyme in the Minitan® reactor.

To summarise it appears likely that both continuous catalytic activity and operational stability were important factors which "enhanced" stabilisation of the enzyme in the Minitan® reactor. Unfortunately their relative contributions to the effect cannot be drawn from this investigation and assessment of their importance would require further experimentation.

In comparison to the results of other workers the total turnover number for NAD^+ (11.72) of the reaction was poor. For example in a similar two-phase systems Lemiere et.al. (1985) reported a TTN of up to 1000. However, in this work the theoretical maximum turnover number for NAD^+ was only 20 (for the first four reactions). Essentially the situation arose because of the low reaction rate of the immobilised enzyme; to avoid very lengthy reaction times octan-1-ol was supplied at 5 mM and this limited the maximum TTN possible. In this light the results appeared more positive and it was considered that with careful selection of experimental conditions (eg. pH) turnover numbers could probably be made to approach the theoretical maximum.

8.4 CONCLUSIONS

1) A small working reactor was constructed in which a model reaction was performed where NAD^+ in an aqueous phase and octan-1-ol in an organic phase remained distinctly separated by a microporous membrane, reaction of these substrates could be catalysed by HLADH immobilised onto the separating

membrane.

2) Reaction rates in the system were approximately 100 fold lower than in a comparative soluble system. Reduction of activity was assigned mainly to loss of catalytic activity of the enzyme on immobilisation.

3) Contrary to the kinetics observed for soluble enzyme kinetic behaviour of the immobilised enzyme could not be described by a simple two substrate Michaelis-Menten expression.

4) Kinetic behaviour of the immobilised enzyme was shown to result from the influence internal mass transfer limitation of the reaction and charge partition effects between the membrane and the aqueous phase substrates. These two effects were shown to be antagonistic to each other. Internal mass transfer limitation was viewed as probably the most significant influence.

5) Reaction was demonstrated reactor which incorporated coenzyme regeneration although the total turnover number of NAD^+ was limited by the low reaction rate of the enzyme.

6) Fractional conversions in the reactor were high (approximately 0.9). This was attributed as a possible benefit of carrying out the reaction in a two-phase system.

7) In the reactor, enzyme stability was enhanced. This was attributed to be a

mixture of operational stability and stabilisation due to continuous catalytic activity.

8.5 AN EXAMINATION OF THE FEASIBILITY OF THE REACTION SYSTEM

It is clear from the literature that in specific cases there is probably a distinct economic advantage in performing a bioconversion in a two phase system or organic media alone. In this thesis the feasibility of using a cofactor dependant enzyme in a two-phase membrane reactor was examined. As the work was unsupported by previous experiments then demonstration of the technical feasibility of the system was the main goal. This goal was achieved in part, importantly, the overall concept of the reactor was demonstrated to be technically feasible, however, the system used suffered from low reaction rates and a low turnover of coenzyme.

The experimental work raised a number of important points for the future design of a similar reactor and the selection of a suitable reaction:

1) The main physical design concern can be identified as the choice of the membrane. An ideal membrane should have a large pore size with maximum pore area. The limitation to this specification being the pore size at which phase mixing becomes unmanageable. Also the membrane should be as thin as possible to limit internal mass transfer restrictions and maximise the reaction rate. The membrane should also be capable of immobilising a large amount of highly active enzyme.

2) The aqueous phase volume can be minimised as it only functions to retain the coenzyme (and possibly the regeneration system).

3) A reaction system where the product hydrophobic and the substrate hydrophillic is the best choice.

4) Reactions converting NADH to NAD^+ are the best choice. This is because coenzyme regeneration strategies for the conversion of NAD^+ back to NADH are more highly developed than NADH to NAD^+ regeneration systems. Formate dehydrogenase catalysed regeneration of NADH from NAD^+ has already been demonstrated to be economically viable in a large scale reactor (Wandrey and Bossow, 1986).

5) Minimisation of external mass transfer by the use of a suitably high flow rates across the membrane is desirable.

6) Use of a membrane with no net charge would be desirable or manipulation of the membrane charge to provide an advantageous partition effect.

To summarise, the work has shown the novel reactor to be feasible in principle but not without problems. From the points outlined above it can be clearly seen that for the reactor to progress toward being economically attractive is as much a question of identifying the right reaction as it is of the physical design of the system.

8.6 PROPOSALS FOR FUTURE WORK.

8.6.1 Selection of a Reaction System

In the light of the recommendations above the first step in any future work should be the identification of a reaction suited to the system. Such a reaction would probably be a chemically difficult organic synthesis where an NADH dependant enzymatic route to the product is available but possibly is unused because of the low solubility of the reactants/products in aqueous media (the NADH dependant steroid transformations examined by Carrea and Cremonesi which were referenced in Section 1.3.1 may provide a suitable starting point). Ideally the enzyme should be highly active, stable and cheap possibly being a whole cell preparation or crude enzyme. It is likely that several candidate reactions will need close examination to identify the most promising one at an early stage.

Regeneration of NAD^+ should be tried using the formate dehydrogenase method. This method is the most highly developed coenzyme regeneration strategy, it can be economically viable and it is simple.

8.6.2 Improvements to the Immobilised Enzyme Environment

A possible method to improve enzyme loading, enzyme activity and reduce mass transfer limitations would be to manufacture "enzyme membranes". This can

be simply accomplished by exposing a solution of enzyme to a protein cross-linking agent such as glutaraldehyde. The mixture obtained can then be spread on glass and dried to produce a thin "enzyme membrane". The pore size can be controlled by the amount of glutaraldehyde added (Trevan 1980). In this way, very porous membranes with high enzyme activity may be produced. Their activity could be tested rapidly using apparatus similar to the small glass reactor.

CHAPTER 9

APPENDICES

APPENDIX 1

THE PROGRAM LISTING FOR THE DATA LOGGER

```
'       ************************************************
'
'            a program to log analogue inputs,
'
'          store, and continuously display the data.
'
'       ************************************************
                    '
dim t(100,8)              '
dim y%(125,8)              '
dim w(1000)               '     dimension arrays
dim comd$(8),b$(8)         '
dim realno(8)             '
dim buffer%(20)            '
cls

print tab (13) " ********d a t a * l o g g e r********"

                    'set up the i/o card and create a file to
                     receive the logged data (if required)

print:print tab (10)
input "do you want to store the data on disk,Y or N";disk$    '
if disk$<>"y" then 10                                          '
print:print tab (10)                                    'open+name
input "name the file please";nme$                            ' disk file
nme$="a:"+nme$                                                '
open nme$ for output as #1
close# 1

                    'input variables

10 print:print tab (10)"enter the total time for the run(hrs,min,secs)"
print:print tab (10)
input "hrs";hours
print:print tab (10)
input "mins";minutes
```

```
print:print tab (10)
input "secs";seconds
if hours >60 or minutes>60 or seconds >60 then print tab (30) "E*R*R*O*R"
tot=(hours*60)+(minutes)+(seconds/60)
print:print tab(10)
input "enter the delay required between scans(in seconds)";x
print:print tab (10)
input "enter the number of channels";nchan

                    'set up each channel and write
                    'command string to i/o card

for j=1 to nchan
   print:print
   print tab (10)
   input "enter channel number";realno(j)
   print:print tab (10)
   input "enter filter number";f$
   print:print tab (10)
   input "enter gain(10,1,0.1)";g

   if g=10 then g$="0"
   if g=1 then g$="1"
   if g=<1 then g$ ="2"

   i$=str$(realno(j))

comd$(j)=chr$(13)+"F"+f$+",G"+g$+",I"+i$+",T"+chr$(13)
   b$(j)=comd$(j)
next j

                    'set up graphics screen +characters

screen 2
view (30,5)-(718,230),0,1
window (0,0)-(125,3400)
circle (60,160),0.05
get (59.7,174)-(60.2,153),buffer%

locate 1,1
print "100"
locate 9,1
print "50"
locate 17,1
print "0"
locate 18,3
print "125"
locate 18,17.5
```

```
print "100"
locate 18,33
print"75"
locate 18,48.5
print "50"
locate 18,65
print "25"
locate 18,80
print "0"

cls
locate 20,1
print "TMINS="
print:print "CHAN"
print "VALUE"
                    'set up array y%( , ) offset to ensure
                    'all graphics characters appear on the
                    'graphics screen

for p=1 to 125
  for q=1 to 8
    y%(p,q)=23
  next q
next p
                   'call data in, write it to both disk file
                   'arrays and graphics arrays

time$="0"
20 for k=1 to 100
   q=timer
   w(k)=q/60
   if q/60 >= tot then 15
   locate 20,8
   print using"####.###";q/60
   for j=1 to nchan
       jj=((j-1)*7)+10
       locate 22,(jj+4)
       print realno(j);
       comd$(j)=b$(j)
       call datin (comd$(j),t(k,j))
       locate 23,jj
       print using "######";t(k,j);
       y%(125,j)=t(k,j)
       if y%(125,j) >= 3400 then y%(125,j)=3400
       if y%(125,j) < =23 then y%(125,j)=23
       for zz= 1 to 124
          put (zz,y%(zz,j)),buffer%
          y%(zz,j)=y%((zz+1),j)
```

```
            put (zz,y%((zz),j)),buffer%,pset
        next zz
    next j
    print
    delay x
next k

                    'open disk file after every 100
                    'values and write them in

15 if disk$ <> "y" then 30
open nme$ for append as #1
for m=1 to 100
print#1,using"#####.###";w(m);
for 1=1 to nchan
print#1,using" ###### ";t(m,l);
  next l
  print#1,
  next m
close #1
30 if q/60 < tot then 20
40 end

                    'subroutine to setup i/o card
                    'to log incoming signals

sub datin(cmd$,D)
local i
dim dt(20)

50 n=len(cmd$)
for i=1 to n
a$=left$(cmd$,1)
cmd$=right$(cmd$,n-i)
dt(i)=asc(a$)
next i

for j=1 to n
st%=128
while (st% and 128) = 128
st%=inp(&h502)
wend
OUT &h500,dt(j)
next j

100 st%=inp(&h502)
if st%<>0 then 100
hibyte=inp(&h501)
```

```
for k=1 to 500
next k
lobyte =inp(&h501)

D=((256*HIBYTE)+LOBYTE)-32768
end sub
```

APPENDIX 2

KINETIC CONSTANTS FOR THE IMMOBILISED ENZYME

Operational Kinetic Constants	Value Obtained
Km NAD^+ μMolar	112
Km Octan-1-ol μMolar	179
Vmax μMoles NADH/min/mg HLADH	0.0295
Ks NAD^+ μMolar	880

Table 5.1 Operational Kinetic Constants of the Immobilised Enzyme.

The constants are described as "operational" do denote their doubtful physical significance and to separate them from apparent values used in the direct linear plot method.

APPENDIX 3

REACTION PROGRESS CURVES FOR THE REPETITVE BATCH REACTIONS IN THE MINITAN® RIG

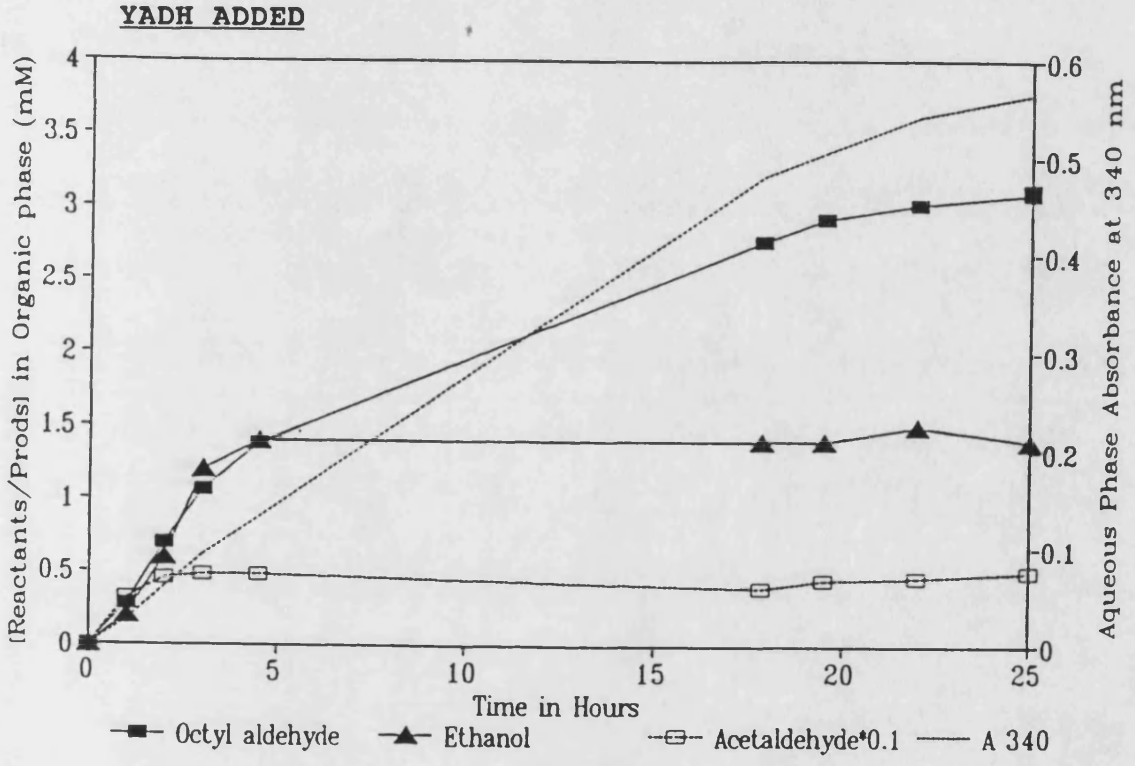

Fig 10.1. First 24 Hour Run in the Batch Reactor

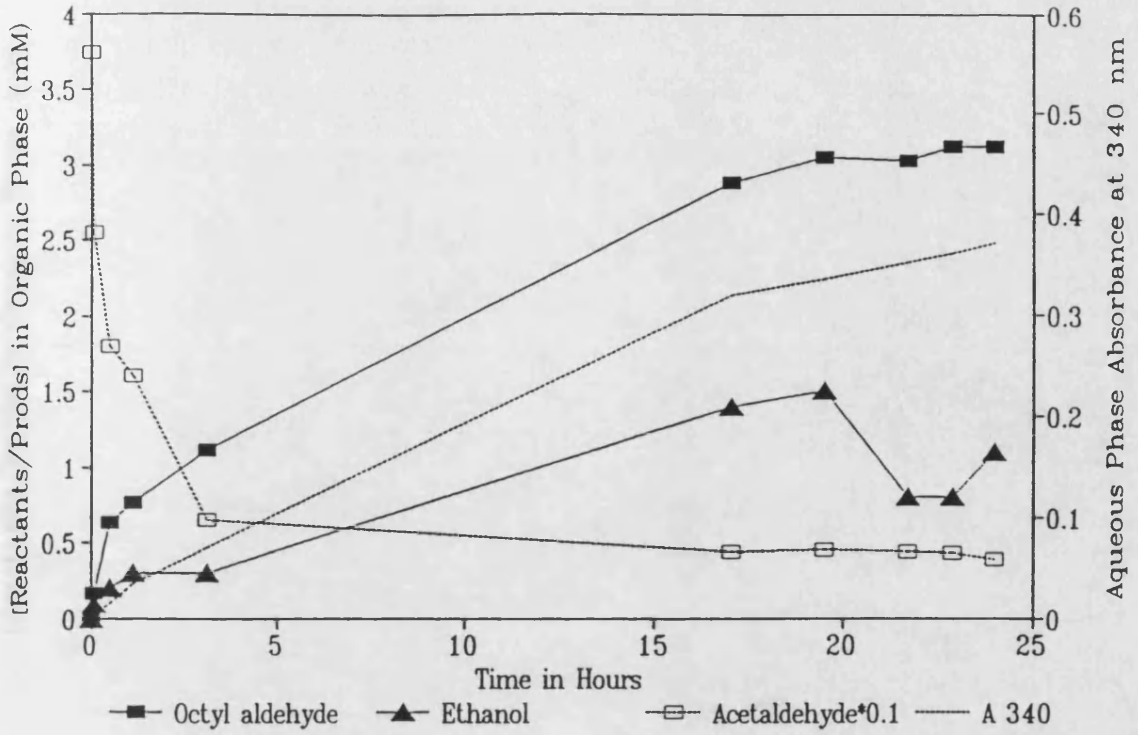

Fig 10.2. The 2nd, 24 Hour Run in the Batch Reactor.

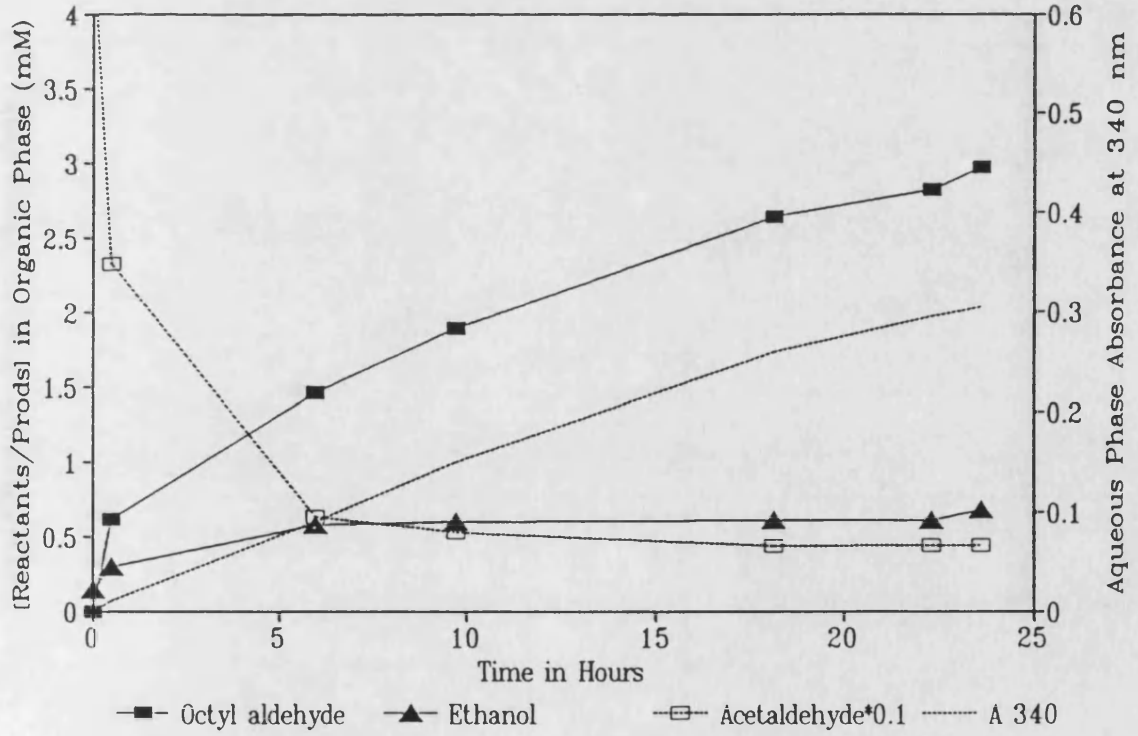

Fig 10.3. 3rd, 24 Hour Run in the Batch Reactor

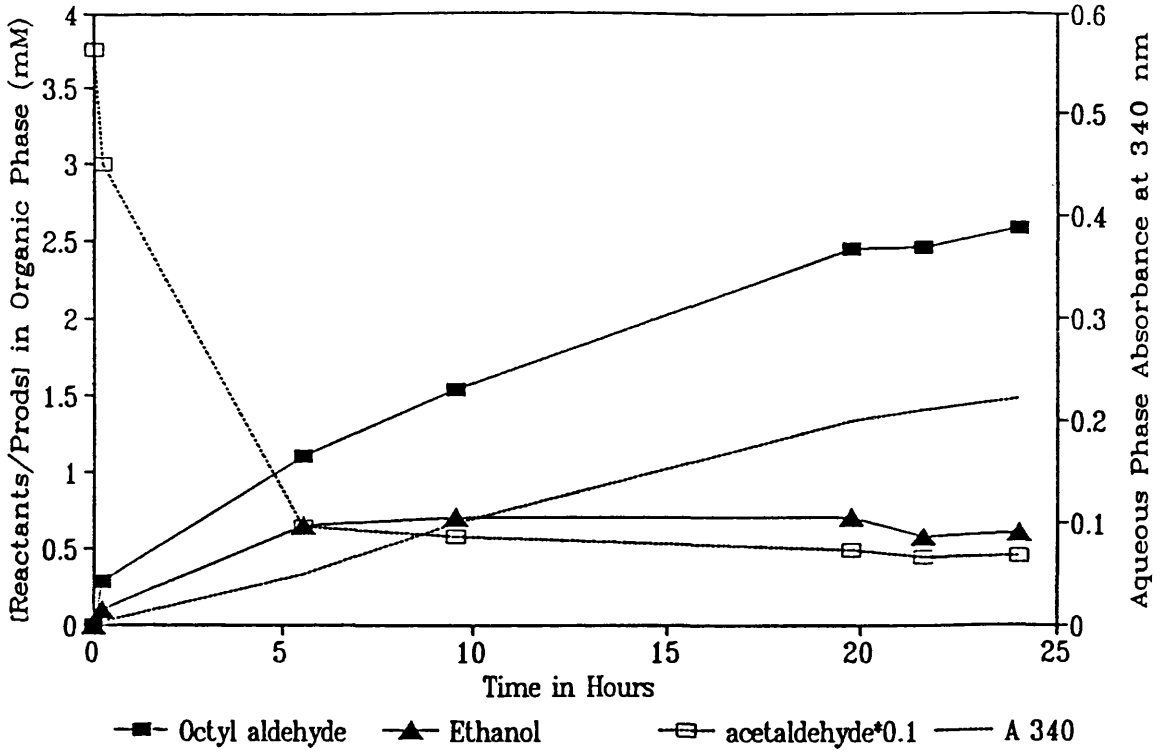

Fig 10.4. 4th, 24 Hour Run in the Batch Reactor

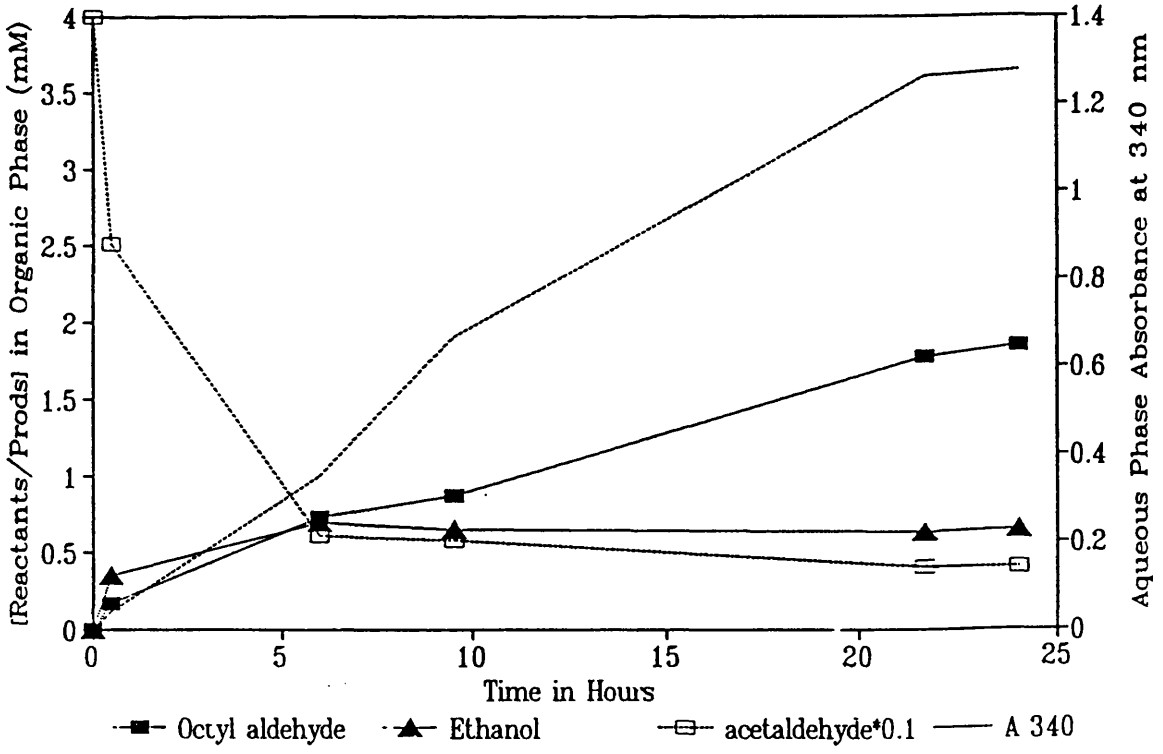

Fig 10.5. 5th, 24 Hour Run in the Batch Reactor (new aqueous phase added)

CHAPTER 10

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